

IMPACT OF NONSTRUCTURAL HEPATITIS C VIRUS ANTIGENS AND TOLL-
LIKE RECEPTOR AGONISTS ON DENDRITIC CELL IMMUNOGENICITY

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Department of Microbiology and Immunology
College of Medicine University of Saskatchewan
Saskatoon, Canada

By

ETHEL ATANLEY

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ABSTRACT

Dendritic cells (DCs) function mainly as antigen presenting cells (APCs) and as such they play a significant role in activating the adaptive immune system. Dendritic cells express toll-like receptors (TLR), and when these receptors are engaged by their cognate agonists, they promote DC maturation, which is critical in the induction of potent T helper (Th) cell -1 responses. Due to the multifunctional abilities of DCs, they have been explored as vaccine carriers, largely in cancer immunotherapy and some infectious diseases including hepatitis C. Previous studies showed that DCs loaded with mRNA of hepatitis C virus (HCV) antigen(s) induced strong immune responses but immune protection was not complete. Therefore, I expected that adoptive transfer of DCs transfected with HCV NS3/4A and/or NS5A mRNA and further treated with TLR agonist(s) *ex vivo* would induce HCV-specific immunity *in vivo*.

Bone marrow-derived DCs generated with Flt3L (FL-DCs) or GM-CSF (GM-DCs), and loaded with HCV NS3/4A and/or NS5A mRNA showed maturation characteristics and produced substantial amounts of IL-12 after *ex vivo* activation with CpG ODN or CpG ODN plus Poly I:C, when compared to their untreated counterparts. Treatment with a combination of CpG ODN and Poly I:C synergized to augment IL-12 production in comparison with stimulation with CpG ODN alone. IL-12 secretion by DCs is pivotal in directing immune responses towards a Th1-bias response, which is needed to eliminate HCV. However, the *ex vivo* responses of stimulated DCs bearing HCV antigen(s) were not efficiently translated in mice to potentiate vigorous antigen-specific T cell responses. This resulted in a lack of protection after challenge with recombinant vaccinia virus expressing HCV NS3/NS4/NS5 in immunized mice.

In contrast, both antigen-specific humoral and cell-mediated immune responses were induced in mice vaccinated with HCV recombinant NS3 or NS5A protein co-formulated with CpG ODN, host defense peptide and polyphosphazene. These responses, however, did not mediate viral clearance, as vaccinated mice remained unprotected from infection with recombinant vaccinia virus expressing HCV antigens. Taken together, these results suggest HCV recombinant protein co-formulated with triple adjuvant to be a better vaccine candidate than the DC-based vaccine.

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DEDICATION

To my mother for inspiring me through the thick and thin of life

TABLE OF CONTENTS

PERMISSION TO USE.....	i
ABSTRACT	ii
ACKNOWLEDGEMENTS.....	iv
DEDICATION.....	v
TABLE OF CONTENTS	vi
LIST OF TABLES.....	x
LIST OF FIGURES	xi
LIST OF ABBREVIATIONS	xiii
1.0 INTRODUCTION AND LITERATURE REVIEW	1
1.1 Hepatitis C	1
1.1.1 Epidemiology and burden of HCV infection.....	1
1.1.2 Virology of hepatitis C virus	2
1.1.2.1 Genome of HCV	2
1.1.2.2 Structural proteins.....	4
1.1.2.3 Nonstructural proteins	7
1.1.3 Interaction of HCV with the immune system.....	11
1.1.3.1 Innate immune responses.....	11
1.1.3.2 Adaptive immunity	13
1.1.4 Treatment and possible vaccine strategies.....	14
1.1.4.1 Current treatments for hepatitis C.....	14
1.1.4.2 Novel HCV therapies in development.....	15

1.1.4.3 Hepatitis C virus vaccines	16
1.2 Dendritic Cells	18
1.2.1 Features and functions of dendritic cells	18
1.2.2 Heterogeneity of dendritic cells.....	20
1.2.3. <i>In vitro</i> generation of dendritic cells.....	22
1.2.4. Loading dendritic cells with exogenous antigen	23
1.2.5. Dendritic cell-based vaccine.....	24
1.3 Toll-like Receptors	25
1.3.1 Dendritic cells and TLRs with their cognate ligands.....	25
1.3.2 Ajuvanticity of TLR ligands and synergy in vaccine formulations.....	27
2.0 HYPOTHESIS AND OBJECTIVES.....	29
3.0 MATERIALS AND METHODS	30
3.1 Generation of bone marrow-derived DCs.....	30
3.2 <i>In vitro</i> maturation of DCs.....	31
3.3 Flow cytometric analysis of surface molecule expression	31
3.4 Isolation of splenocytes	32
3.5 T cell isolation by magnetic separation with LS columns.....	33
3.6. Mixed lymphocyte reaction	34
3.7 <i>In vitro</i> transcription (IVT) of mRNA.....	34
3.8 Transfection of DCs.....	35
3.9 Immunocytochemistry	35
3.10 Cytokine ELISA	36
3.11 Production of HCV recombinant proteins	36

3.12 Cell line, propagation and purification of recombinant virus.....	40
3.13 Immunization and virus challenge of mice.....	41
3.14 IFN- γ and IL-5 ELISPOT assays.....	42
3.15 Intracellular cytokine staining	43
3.16 Serum antibody ELISA	43
3.17 Protection and plaque assay.....	44
3.18 Statistical analysis.....	45
4.0 RESULTS.....	46
4.1 Phenotypic characteristics of FL-DCs and GM-DCs	46
4.2 Influence of TLR agonist treatment on FL-DCs and GM-DCs.....	50
4.2.1 Combination of CpG ODN and Poly I:C augments IL-12p70 production.....	50
4.2.2 Allostimulatory capacity of FL-DCs	54
4.3 <i>Ex vivo</i> antigen loading of FL-DCs and GM-DCs.....	56
4.4 Functional characteristics of FL-DCs bearing HCV NS3/4A or NS5A used for vaccination.....	59
4.5 Adaptive immune responses and protection following immunization with FL-DCs transfected with HCV NS3/4A or NS5A mRNA.	63
4.6 Effect of route of administration and combination of CpG ODN and Poly I:C on immune responses after vaccination with FL-DCs transfected with HCV mRNAs.	65
4.7 Adoptive transfer of GM-DCs loaded with single or combined HCV mRNA(s) and activated with TLR-L(s)	70
4.8 CpG ODN-activated GM-DCs carrying HCV antigen secrete IL-5.....	76

4.9 HCV-specific immune responses induced by HCV recombinant NS3 or NS5A protein formulated with triple adjuvant.	78
5.0 DISCUSSION AND CONCLUSIONS	81
5.1 Impact of TLR agonist(s) treatment on the phenotypes of FL-DCs and GM-DCs .	81
5.2 HCV-specific immune responses induced by DC-based vaccination	83
5.3 Cellular and humoral responses to HCV recombinant protein vaccine.....	87
5.4 General conclusion and future directions	90

LIST OF TABLES

Table 3.1. Buffers used in protein purification.....	39
Table 4.1. Transfection efficiency and viability of FL-DCs 24 h post transfection with GFP mRNA.....	57

LIST OF FIGURES

Figure 1.1. Replication cycle of hepatitis C virus.	4
Figure 1.2. A summary of TLR signaling pathways indicating receptor ligands, adaptor molecules and the responses induced.	27
Figure 3.1. Identification of purified HCV recombinant proteins.	39
Figure 4.1. Differential expression of surface proteins by FL-DCs and GM-DCs.	47
Figure 4.2. Morphology of FL-DCs and GM-DCs.	48
Figure 4.3. TLR ligand-treated FL-DCs and GM-DCs have elevated expression of maturation markers.	49
Figure 4.4. Stimulation with TLR-L(s) induces cytokine secretion by FL-DCs and GM-DCs.	51
Figure 4.5. Combination of CpG ODN and Poly I:C does not affect the maturation phenotype of FL-DCs or GM-DCs.	53
Figure 4.6. TLR ligand treated FL-DCs induce allogeneic T cell proliferation.	55
Figure 4.7. FL-DCs and GM-DCs loaded with GFP mRNA.	58
Figure 4.8. CpG ODN-activated FL-DCs <i>ex vivo</i> prior to adoptive transfer.	60
Figure 4.9. Expression of HCV NS5A by FL-DCs.	62
Figure 4.10. Recombinant vaccinia virus titer in challenged mice after immunization with FL-DCs bearing HCV antigens.	64
Figure 4.11. Cytokine secretion and allostimulatory capacity of CpG-ODN and Poly I:C treated FL-DCs carrying HCV antigens.	66
Figure 4.12. HCV-specific cell-mediated immune response and viral load following vaccination with HCV antigen-bearing FL-DCs.	69

Figure 4.13. Impact of TLR-L(s) activation on GM-DCs used for adoptive transfer	71
Figure 4.14. Immunocytochemistry of GM-DCs expressing HCV antigen	73
Figure 4.15. Effect of TLR-L(s) and HCV antigen(s) on adaptive immune responses and protection by GM-DC-based vaccination	74
Figure 4.16. IL-5 and IFN- γ production in DC cultures and DC-T cell cocultures	77
Figure 4.17. HCV-specific immune responses and viral load in mice vaccinated with rNS3 or rNS5A	79

LIST OF ABBREVIATIONS

AP	Alkaline phosphatase
APC	Allophycocyanin
APCs	Antigen presenting cells
BMDCs	Bone marrow-derived dendritic cells
BSA	Bovine serum albumin
BSC-1	African green monkey kidney epithelial cells
CD	Cluster of differentiation
CTL	Cytotoxic T lymphocytes
CPM	Counts per minute
CpG ODN	Cytosine-phosphate-guanosine oligodeoxynucleotides
cDC	Conventional dendritic cell
DC	Dendritic cell
ds	Double-stranded
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunospot assay
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FL-DCs	Bone marrow-derived dendritic cells generated with Flt3L
Flt3L	Fms-like tyrosine kinase 3 ligand
FITC	Fluorescein isothiocynnate
GM-DCs	Bone marrow-derived dendritic cells generated with GM-CSF
GM-CSF	Granulocyte macrophage-colony stimulating factor
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HDP	Host defense peptide
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
i.p	Intraperitoneally

kDa	Kilo dalton
LPS	Lipopolysacchacride
MEM	Minimum essential medium
MHC	Major histocompatibility complex
min	minute
MLR	Mixed lymphocyte reaction
MOI	Multiplicity of infection
mRNA	Messenger ribonucleic acid
NS	Non-structural
PBS	Phosphate buffered saline
PCEP	Poly [di(sodium carboxylatoethylphenoxy)- polyphosphazene]
PE	Phycoerythrin
pDC	Plasmacytoid dendritic cell
PFU	Plaque-forming unit
p.i	Post infection
Poly I:C (PIC)	Polyinosinic:polycytidylic acid
PRR	Pattern recognition receptor
PAMP	Pathogen-associated molecular pattern
rNS3	Recombinant nonstructural 3
rNS5A	Recombinant nonstructural 5A
rVV	Recombinant vaccinia virus
s.c	Subcutaneously
SEM	Standard error of the mean
STAT	Signal transducer and activator of transcription
SDS-PAGE	Sodium dodecyl sulphate-poly acrylamide gel electrophoresis
Th	T helper cell
Th1	T helper-1 cell
Th2	T helper-2 cell
TLR	Toll-like receptor
TLR-L	Toll-like receptor ligand
TNF	Tumor necrosis factor

1.0 INTRODUCTION AND LITERATURE REVIEW

1.1 Hepatitis C

1.1.1 Epidemiology and burden of HCV infection

Hepatitis C virus (HCV) is a blood-borne infectious agent responsible for the cause of hepatitis and chronic liver disease [1]. The hepatitis C virus has been reported to infect an estimated 2-3% of the world's population and it is also the reason for most liver transplants in many of the developed countries [2-4]. There are 3 to 4 million new HCV infections and over 350,000 deaths due to hepatitis C-related liver diseases that occur each year [5, 6]. The epidemiology and burden of HCV infection vary widely geographically. According to the public health agency of Canada, in 2007 about 242,500 individuals in Canada were infected and close to 8000 new HCV incidences were reported that same year [7]. Unlike Canada and some other countries with less than 2% prevalence rate, a country like Egypt has been reported to have the highest prevalence rate of 15-20% of anti-HCV positive individuals among its general population: making HCV a global health concern [8, 9].

An HCV-infected individual can either develop an acute or chronic infection. Acute HCV infection is most often asymptomatic, and only a small fraction of patients with this infection may show some clinical signs. Approximately 75% of acute hepatitis C infections become chronic due to persistent viremia, with spontaneous HCV clearance seen in up to 25% of the acute HCV infections [10, 11]. Of the proportion of chronically HCV-infected patients, 10-20% of them stand the risk of developing cirrhosis and 1-5% of the cirrhotic infections may progress to liver cancer or hepatocellular carcinoma many

years later [8, 12-14]. The growing rate of HCV spread mostly is tied to the various routes of transmission. Hepatitis C virus is contracted generally through contact with infected blood products. For example, the high prevalence rate of hepatitis C in Egypt now is the result of past country-wide efforts to control schistosomiasis, where needles were unsterilized or reused to administer treatment (reviewed in [15]). In many developed and some developing countries, HCV transmission via transfusion has dramatically declined since the introduction of screening donor blood for antibodies against HCV. Unfortunately, post-transfusion hepatitis C continues to remain a major risk factor in other developing countries [16-18]. In recent times, HCV transmission through intravenous use of illegal drugs has been common, as a proportion of these drug users tend to share injection needles [19]. Further, HCV can be spread through sexual contacts. High incidence rates have been reported among homosexuals and even more so in individuals with HIV-positive partners, respectively [20-22].

1.1.2 Virology of hepatitis C virus

1.1.2.1 Genome of HCV

Hepatitis C virus is an enveloped positive sense single-stranded RNA virus. HCV belongs to the genus *Hepacivirus* and it is a member of the *Flaviviridae* family. The 30-80 nm-sized hepatitis C virion [23, 24] has its genomic RNA enclosed in an icosahedral capsid and an envelope with two distinct glycoproteins [25]. The HCV genomic RNA has a single open reading frame (ORF) of about 9.6 kilobases that is flanked by relatively conserved untranslated regions (UTR) at the 5' and 3' ends [26]. Available data show that the 5' UTR contains an internal ribosome entry site (IRES), required for the initiation

of cap-independent translation of the virus genome. The genomic RNA of HCV yields a polyprotein of approximately 3000 amino acids upon translation [27, 28]. The viral polyprotein at the immature stage is cotranslationally and posttranslationally processed by viral and host proteases into 10 different mature proteins, three of which are structural proteins (core, envelope glycoproteins E1 and E2) released by host cell signal peptidases. The remaining seven proteins encode the nonstructural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, NS5B) and are released by viral proteases [29, 30].

Hepatitis C virus mainly infects and replicates in human hepatocytes. However, other cells like DCs, B cells and monocytes have been demonstrated to harbor the virus as well [31, 32]. Figure 1.1 shows the HCV replication cycle. To initiate infection, the viral envelope glycoproteins E1 and E2 attach themselves to several cellular receptors to gain entry into the host cell. Upon entry, the viral nucleocapsid is released into the host cell cytoplasm following clathrin-mediated endocytosis accompanied by endosomal acidification, which trigger the fusion of the viral glycoproteins with the endosomal membrane [33-35]. The genomic RNA of HCV is next decapsidated into the cytosol, translated and processed into individual proteins. In a replication complex consisting of cellular and viral proteins, the viral RNA-dependent RNA polymerase utilizes the viral genomic RNA as a template for synthesis of a complementary negative strand RNA which is then further synthesized into a positive strand RNA in the HCV replication cycle [36, 37]. Core binds to the newly synthesized genomic RNA and acquires its envelope by budding through endoplasmic reticulum (ER) membranes [38]. The formed HCV particles then exit the host cell via the secretory pathway [39].

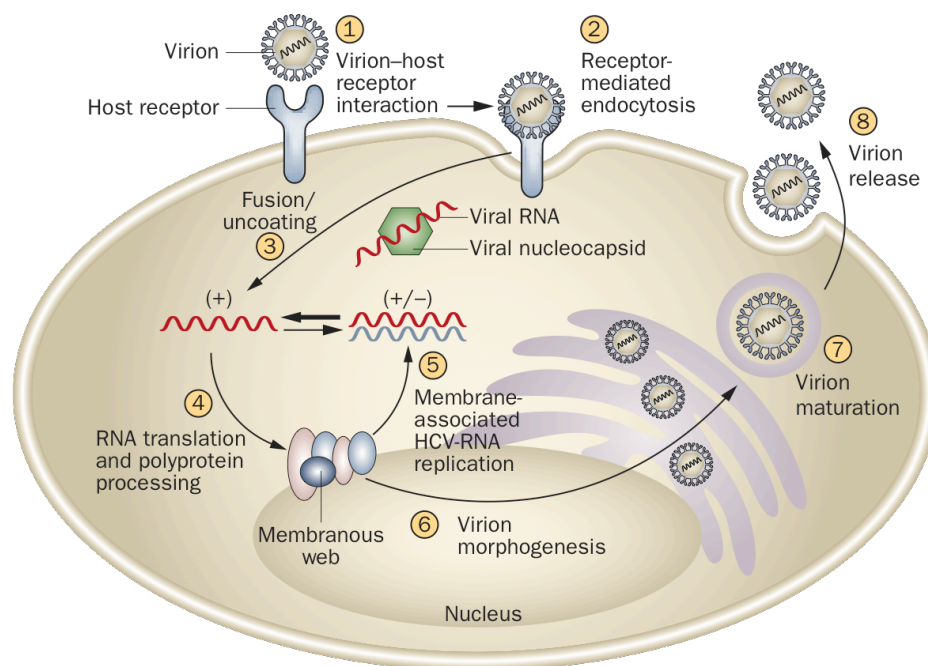


Figure 1.1. Replication cycle of hepatitis C virus [40].

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The RNA-dependent RNA polymerase is error prone and lacks a proofreading activity (reviewed in [41]). Thus, HCV clones from infected individuals have been reported to show striking genetic heterogeneity, therefore making the virus exist as quasispecies. Currently, six major HCV genotypes comprising of several subtypes have been recognized [42].

1.1.2.2 Structural proteins

Core protein

Hepatitis C virus core protein is a highly basic protein, positioned at the amino terminus of the nascent viral polyprotein. Unlike the nonstructural proteins, the 21

kilodalton (kDa) core protein encoded by 191 amino acids (aa) is released by cellular proteases during proteolytic processing of the viral polypeptide [29, 43]. Core protein is thought to form the viral nucleocapsid and it possesses three definite functional domains. The first 122 aa encode the amino-terminal hydrophilic domain 1, which functions largely in RNA binding and core oligomerization [44, 45]. Domain 2 is, however, hydrophobic in nature and is located between 123-174 aa of the core protein. The latter is involved in the association of core with the ER membrane and lipid droplets, and remains an indispensable component for efficient HCV assembly [43, 46, 47]. The highly hydrophobic domain possibly positioned between 175-191 aa of the core protein acts as the signal peptide for translocation of the E1 protein into the ER lumen [43, 45]. In addition to the functions of core in the life cycle of HCV, the core protein is capable of influencing several cellular processes. The involvement of core in recruiting HCV RNA, nonstructural proteins and replication complex to lipid droplet-associated membranes required for virion assembly [45] has been implicated in chronic HCV-associated steatosis. Core causes excessive lipid droplet production by possibly binding to enzymes in the lipid metabolism leading to huge lipid droplets in liver cells [48]. Furthermore, core is able to induce apoptosis in lymphoid and liver cells during the initial stages of synthesis and processing of the protein [49, 50]. Moreover, core may be engaged in regulating transcription and the proliferative capacity of host cells, which has been correlated to hepatocarcinogenesis [51-53]. Modulation of cellular immune responses including the suppression of suppressor of cytokine signaling (SOCS) by core may assist in the development of HCV-associated liver cancer [47, 53].

Besides HCV core protein, an alternative reading frame protein (ARFP) is translated from the reading frame of core protein due to a -2/+1 ribosomal frameshift [54]. Translation of the 17 kDa ARFP has been reported to be IRES-independent [55, 56], and although the function of this protein has not been elucidated, it has been proposed that ARFP may be involved in the virus life cycle [54].

Envelope glycoproteins E1 and E2

The envelope proteins of HCV are type 1 transmembrane glycoproteins and like core protein, are released after cleavage by signal peptidase during HCV polyprotein processing. This yields E1 and E2 proteins with apparent molecular weights of 35 and 70 kDa, respectively [57-60]. Both E1 and E2 glycoproteins possess an amino-terminal ectodomain and carboxy-terminal transmembrane domain. In addition, E1 and E2 proteins have 5 and 11 amino-linked glycosylation sites, respectively [61, 62], and they assemble mostly as non-covalent heterodimers in the ER and transit through the secretory pathway [63, 64]. Like for many enveloped viruses, the envelope glycoproteins E1 and E2 of hepatitis C virus are required to initiate the life cycle of hepatitis C virus. These structural proteins of the virus are actively involved in the entry of the virus into host cells during the early stages of HCV infection [57, 65]. The E2 glycoprotein is involved in direct HCV attachment to several membrane receptors including cluster of differentiation (CD) 81 [66], scavenger receptor class B type 1 [67], occludin [68] and claudin-1 [69], low-density lipoprotein receptor [70], glycosaminoglycan [71], dendritic cell-specific intracellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN) or liver/lymph-specific intracellular adhesion molecule 3-grabbing integrin (L-SIGN) [72]

prior to virus entry. Approximately 26 or 27 amino-terminal aa of the E2 protein contain a hypervariable region 1(HVR1), the most variable sequence in the HCV genome, with a stretch of positively charged residues located in a specific position of this region [73, 74]. Notably, the basic residues have been shown to contribute to HCV entry [75]. Also, epitopes in the HVR1 of E2 protein can trigger neutralizing antibodies following HCV infection [76]. Genetic heterogeneity of HVR1 of the E2 protein accounts for the heterogenous forms and immune escape of HCV [77, 78]. This may not be surprising as E2 protein plays a more direct role in virus attachment than E1 protein. Besides the involvement of E1 glycoprotein in HCV entry and host membrane fusion, not much is known about its distinct role in HCV infection [79].

1.1.2.3 Nonstructural proteins

p7 is a small hydrophobic protein of 63 amino acids in length [80]. This double membrane spanning protein has a molecular mass of 7 kDa [80, 81]. According to several reports, p7 forms ion channels [82, 83] like HIV-1 viral protein U and matrix protein 2 of influenza A virus. p7 has been demonstrated to act as a bona fide viroporin [84, 85]. Thus, this viral protein participates in virus assembly and egress of the infectious HCV particles, and hence may be involved in the late stages of the viral replication cycle [84]. However, p7 has also been shown to be important for HCV infectious virion production in the early stages of the viral life cycle [86]. Further studies have also revealed that p7 is essential for the virus infectivity and possesses sequences with genotype-specific functions [87], and it is therefore a suitable target for antiviral-therapy [83, 85, 88].

Nonstructural protein 2 (NS2)

NS2 is 23 kDa transmembrane protein processed by a zinc-dependent NS2-NS3 cysteine protease, a region of the HCV genome that encodes NS2 protein, the NS2/3 cleavage site and the serine protease domain of NS3 [89-91]. Upon the autoproteolytic cleavage of the NS2-NS3 junction, NS2 protein is integrated into ER membrane [92]; however, the protein is short lived as it gets degraded by proteasomes following phosphorylation by casein kinase 2 [93]. Thus, the processed nonstructural protein 2, but not unprocessed NS2-NS3, has been demonstrated to be involved in the early stages of viral particle production [86]. NS2 contributes to HCV virion assembly by mediating interactions between the HCV structural and nonstructural proteins [90, 94]. Apart from the role of NS2 in the HCV life cycle, NS2 inhibits cytokine gene expression, and it is able to bind liver specific cell death-inducing DFFA-like effector b (CIDE-B) to inhibit CIDE-B induced apoptosis, a possible mechanism HCV uses to evade host cell defenses [95, 96].

Nonstructural protein 3 (NS3)

Hepatitis C virus NS3 is a multifunctional protein with an apparent molecular weight of about 70 kDa [29, 97]. NS3 protein possesses serine protease, nucleoside triphosphatase (NTPase) and RNA helicase activities. One third of the N-terminal region of NS3 contains the serine protease that cleaves NS3/4A, NS4A/4B, NS4B/NS5A and NS5A/5B junctions of the HCV polyprotein and requires the C-terminal portion of the NS4A protein for this activity. The C-terminal region of NS3 encodes the NTPase/RNA helicase [98-102]. There are data suggesting that the RNA helicase of NS3 interacts with

the HCV NS5 proteins to initiate the synthesis of plus strand HCV RNA [103, 104]. The serine protease and helicase domains of NS3/4A have been shown to depend on each other for maximum activity during the virus life cycle [105]. The NS3/4A protease has also been shown to disrupt cytokine-inducing pathways that play critical roles in host antiviral responses [106, 107]

Nonstructural protein 4 (NS4)

The NS4 protein is a hydrophobic protein, which, when processed by NS3-4A protease, yields NS4A and NS4B with apparent molecular weights of 8 and 27 kDa, respectively [29, 100].

NS4A protein forms a complex with the NS3 protease domain and acts as cofactor for NS3 protease [108]. In addition to enhancing the serine protease and helicase activities of NS3 [100, 109, 110], the acid domain of NS4A protein contributes to HCV replication and assembly [111]. The NS4B protein on the other hand is an integral membrane protein that gets associated with the ER membrane during translation [112]. The NS4B protein is known to induce morphological alterations in the ER membrane triggering the formation of structures that represent preferential sites for HCV replication [113, 114]. Further, NS4B is required for hyperphosphorylation of NS5A [115], but suppresses the activity of HCV RNA-dependent RNA polymerase through inhibition of the NTPase domain of NS3. Thus, NS4B may be involved in the regulation of the amount of the HCV genomic RNA produced by the viral polymerase during HCV replication [103]. Both NS4A and NS4B have been implicated in the inhibition of host

cell protein synthesis via translation of NS4A and in addition, suppress cell proliferation [116] and cause apoptosis of HCV-infected cells [117].

Nonstructural protein 5 (NS5)

The NS5 region of the HCV genome encodes NS5A and NS5B proteins. NS5A is a phosphorylated zinc metalloprotein with an apparent molecular weight of 56-58 kDa [118, 119] and three RNA-binding domains [120]. Domain I of NS5A has a zinc-binding motif [118], and domain II is involved in the repression of interferon (IFN) induced-protein kinase R (PKR) activity by binding to PKR in an ISDR (IFN sensitivity determining region)-dependent manner to subvert the host antiviral response [121]. The requirement of domain III of NS5A for HCV viral particle assembly has also been reported [122]. Furthermore, NS5A has been demonstrated to activate NS5B during the elongation phase of replication and the synthesis of negative strands of the HCV RNA genome [123].

The NS5B gene encodes the RNA-dependent RNA polymerase. It has a molecular mass of about 68 kDa [29] and initiates the synthesis of new viral RNA [124]. Variability in the NS5B gene sequence has been documented to be sufficient for accurate classification of HCV genotypes and subtypes [125]. NS5B is further known to interact with host proteins, which either positively or negatively alter the activity of the viral polymerase [126-128]. In contrast to NS5A, it has been indicated that NS5B strongly activates IFN- β via the TLR3/TRIF (TIR-domain containing adaptor protein for inducing IFN- β) signaling pathway and synthesis of double-stranded (ds) RNA [129].

1.1.3 Interaction of HCV with the immune system

1.1.3.1 Innate immune responses

The host immune system is responsible for fighting and eliminating infections. The immune system is capable of generating an innate immune response, which represents the first line of defense. An infected host quickly tries to clear the infection by mounting an innate response, followed by an adaptive response. In case the host immune system is incapable of overcoming the infection, an environment is created for the pathogen to survive for an extended period of time or even a whole life-time if not somehow controlled. This type of situation occurs after HCV infection. As mentioned earlier, HCV induces both acute and chronic infections. The immune system is able to fight HCV infection in about 25% of infected patients at the acute phase of infection [11]. Acute HCV infection is mostly asymptomatic [130]; however, robust and long-lasting cytotoxic T cell (CTL) responses are thought to be required for resolution of this infection [131]. How the immune system recognizes HCV and the mechanisms the virus employs to elude the immediate and adaptive defenses to persist in most individuals, is reviewed in this section.

Innate immune cells including DCs and macrophages express pattern recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs) to stimulate innate immune responses [132]. Subsequently, these cells then signal through adaptor proteins to induce activation of transcription factors such as IFN regulatory factors (IRF) and nuclear factor (NF)- κ B, which further enhance the expression of IFN-stimulated genes (ISGs) [133]. During replication, HCV like many RNA viruses induces the production of ds RNA in the host cells [134], which is detected by two PRRs; TLR3

and RNA helicase retinoic acid inducible gene 1 (RIG-1) [135-137]. In addition, the UTR of HCV RNA has been reported to serve as PAMP that binds RIG-I and thus activates IRF-3 [137]. Both RIG-I and TLR3 signaling restricts HCV replication by activating IRF-3 and inducing the expression of ISGs [137, 138].

HCV is highly prolific; 10^{12} hepatitis C virions are produced in a day [139] with an estimated mutation rate of 2.5×10^{-5} per nucleotide [140], a major means exploited by the virus to escape recognition by the immune system. There are several emerging data implicating HCV in surmounting the innate immune responses to persist, some of which were earlier reviewed in sections 1.1.2.2 and 1.1.2.3. In addition, it has been shown that HCV NS3/4A serine protease can cleave Toll/interleukin-1 receptor (TIR) domain-containing adaptor inducing IFN- β (TRIF) [141] and the caspase activation and recruitment domains adaptor inducing IFN- β (CARDIF) (also known as mitochondrial antiviral signaling protein (MAVS), IFN- β promoter stimulator 1 (IPS-1) or virus-induced signaling adaptor (VISA)), which are adaptor proteins for TLR3 and RIG-I, respectively [142, 143]. HCV core has been reported to cause CD14 monocytes to secrete IL-10 and tumor necrosis factor alpha (TNF- α) which inhibit the production of the host antiviral cytokine, IFN- α , by plasmacytoid DCs (pDCs) [144]. Furthermore, DCs infected with HCV structural proteins have reduced expression of major histocompatibility complex (MHC) class 1 molecules during maturation and diminished ability to activate T cells [145]. The latter has been associated with enhanced IL-10 being secreted by DCs of HCV patients; DCs pulsed with HCV NS3 protein show similar characteristics [146]. On the contrary, the findings of some investigators indicate that DC maturation and function are not impaired in HCV-infected individuals [147, 148]. Also,

the numbers of natural killer (NK) cells representing non-specific IFN- γ secreting cells, and the CTL population of the innate immune system are reduced in seropositive HCV patients [149, 150]. In addition, NK cell subsets that express perforin were found to express lower levels of perforin in infected individuals [151]. Further, HCV core binds to the receptor of the C1q complement protein (gC1qR) expressed on the cell surfaces of monocytes/macrophages and down-regulates IL-12 and T cell proliferation [152, 153].

1.1.3.2 Adaptive immunity

Responses of the innate arm of the immune system particularly those of DCs are essential for shaping the responses elicited during adaptive immunity. In addition to affecting innate immunity, HCV is able to interfere with acquired immunity, which also is a strategy the virus uses to persist in its host.

HCV-specific antibody responses are detected about 6 weeks following exposure to the virus [154]. Some chronically infected HCV individuals and a minority with acute infection develop high amounts of neutralizing antibodies (nAbs). Nonetheless, this response is delayed and mostly not related to HCV clearance in patients with acute or chronic infections [155]. However, nAbs may play a part in the outcome of HCV infection of individuals that clear the infection spontaneously [156]. HCV can interfere with humoral responses through induction of non-nAbs. These non-nAbs bind to epitopes in the hypervariable region of the HCV E2 protein involved in HCV neutralization, thus blocking the activities of the nAbs against the virus [157].

In addition to humoral responses, T cells responses are indispensable in the resolution of many intracellular infections. The involvement of both robust HCV-specific

IFN- γ -secreting Th cells and durable CTL responses is critical in the clearance of HCV RNA [131]. Furthermore, the numbers of HCV-specific CD4⁺ Th1 cells were lower in chronic HCV patients compared to individuals who had resolved the virus infection, emphasizing the importance of cellular immune responses in HCV clearance [158]. In addition, selection pressure exerted by T cell responses against HCV leads to mutation in CD8⁺ T cell epitopes. These altered epitopes have diminished antigen processing, presentation and MHC class I binding, and eventually evade T cell recognition [159]. Some mutant T cell epitopes of HCV also tend to have poor inherent immunogenic properties and as such fail to efficiently prime naïve T cells; another strategy HCV explores to subvert T cell recognition [160]. In the absence of mutational escape of HCV-specific T cell epitopes, the T cells elevate expression of the receptor PD-1 (programmed death-1) [161]; this receptor is an inhibitor of T cell function [162]. Altogether, HCV has evolved to elude almost all arms of the immune system to thrive in its host.

1.1.4 Treatment and possible vaccine strategies

1.1.4.1 Current treatments for hepatitis C

Not until 2011, the combination therapy of pegylated IFN- α and ribavirin (PR) was the standard of treatment for HCV infections. Depending on the infecting HCV genotype, about 42% (genotype 1) to 80% (genotypes 2 and 3) of the patients achieve a sustained virologic response (SVR) following a 48-week treatment regimen. Besides the length of time it takes a patients to attain SVR, the treatment is associated with several

side effects such as neutropenia, anaemia and thrombocytopenia among others [163]. Recently, the United States Food and Drug Administration approved the use of two new NS3/4A protease inhibitors, boceprevir and telaprevir. The combination of either of these protease inhibitors with PR in a triple combination therapy results in more than 70% of individuals infected with HCV genotype 1 attaining SVR. Although this current therapy is an improvement on the previous treatment with low SVR rates in patients with HCV genotype 1, the regimen is also associated with serious adverse effects similar to the PR treatment and possible emergence of drug resistant mutants [164, 165]. Also, the cost of treatment is very high: PR costs range from \$17,175 to \$34,349, telaprevir without PR costs \$49,200 and boceprevir without PR is \$26,410 to \$48,418 depending on the length of therapy [166].

1.1.4.2 Novel HCV therapies in development

In addition to the current therapies for HCV infection, a number of antiviral drugs have been developed with a significant fraction of these drugs in human clinical trials. The different stages, cellular and viral components involved in the HCV life cycle are targets for the novel therapies in development. Besides controlling the virus, some of the treatments are also focused on reducing daily dosing and the duration of therapy with improved tolerability [167, 168]. Among the promising agents developed are inhibitors of NS3/4A protease (danoprevir and vaniprevir), NS5B RNA-dependent RNA polymerase, NS5A, cyclophilin A, microRNA 122 (miR122) and HMG-CoA (3-hydroxy-3-methylglutaryl-coenzyme A) reductase (reviewed in [168]). In addition, small interfering RNAs (siRNAs) targeting HCV proteins are also being tested [169].

Combinations of inhibitors of HCV NS3 protease, NS5B and NS5A replication complex with or without PR in a triple or quadruple therapy are also being studied and have shown improved SVR [168, 170, 171]. The outcomes of these therapeutic agents so far give new hope for superior treatment of HCV infections in the near future. Nonetheless, one major concern with antiviral drugs is the development of drug-resistant variants when used alone during treatment. In both boceprevir and telaprevir monotherapy, HCV variants emerged but when combined with PR, the growth of resistant variants was minimized [172, 173]. The pharmacological properties, target and mode of action of antiviral agents among other factors, determines the level of viral resistance. For instance, protease inhibitors and non-nucleoside HCV polymerase inhibitors have a low barrier to resistance. Nucleoside HCV polymerase and NS5A inhibitors on the other hand induce high and moderate levels of resistance, respectively [168]. Thus, the choice of antiviral agents and combination therapy of PR with future antiviral agents may be important in preventing drug resistance.

1.1.4.3 Hepatitis C virus vaccines

During the search for a HCV vaccine over the years, many strategies have been explored, but despite these efforts, there is still no HCV vaccine licensed or approved for public use. The quest for a HCV vaccine remains despite the advances achieved in development of antiviral drugs against HCV infection. The latter treatments, as mentioned earlier, are very expensive and are also accompanied by various side effects. Therefore, vaccines that will prevent HCV infection and/or treat the virus infection in patients who fail to respond to current therapies are urgently needed. However, progress

in the development of a successful HCV vaccine has been hampered largely by the genetic variability of HCV, but to some extent also due to the lack of suitable animal models for preclinical trials. The animal models available include chimpanzees, tupaia *belangeri*, genetically humanized mice and mice with a chimeric human liver. The chimpanzees are expensive and not readily accessible, and the use of the small animals models are also limited by their low level of viraemia, lack of persistent viraemia or immunodeficiency (reviewed in [174]). Thus, several studies are based on recombinant vaccinia virus encoding HCV genes as an infection model in mice [175-177], while in others tumor cells expressing the HCV antigens are used [178, 179] to assess vaccine efficacy. Furthermore, the induction of transient expression of HCV RNA in mouse hepatocytes via hydrodynamic injection of the desired HCV plasmid DNA can also be used as a model [179, 180]. These surrogate models require efficient CTL responses to control viral infection or tumor growth. Impressively, very recently, Kapoor *et al.* [181] identified HCV-like viruses in deer mice. This significant discovery may help surmount most of the challenges encountered during vaccine testing at the preclinical level in the future.

Most of the vaccines against HCV currently in clinical trials are recombinant proteins, peptides, viral vectors, DNA and DC-based vaccines [182]. Several of the approaches are concentrated on the more conserved HCV core and NS proteins as the target antigens; these proteins also contain CD4⁺ and CTL epitopes [182-184]. HCV vaccines based on the envelope proteins that induce cross-neutralizing antibodies against other HCV genotypes are also in development [185].

1.2 Dendritic Cells

1.2.1 Features and functions of dendritic cells

Dendritic cells represent one type of innate immune cells. They were first discovered by Paul Langerhans in 1868 as Langerhans cells, which represent one class of DCs. However, the term “dendritic cells” was initially used by Ralph M. Steinman and Zanvil A. Cohn in 1973 when they described a cell type found in peripheral lymphoid organs of mice [186]. Dendritic cells are well known for their ability to efficiently present antigens to cells of the adaptive immune system. This role of DCs is important in linking the innate and adaptive arms of the immune system.

Dendritic cells originate from either common myeloid or common lymphoid progenitors of hematopoietic stem cells in the bone marrow [187]. Based on the knowledge obtained from experimental results, DCs derived from their progenitors are considered “immature”. Immature DCs at this stage of life are proficient in capturing antigens and therefore traffic peripheral and lymphoid tissues in search of foreign antigens. Besides foreign antigens, immature DCs also pick up self-antigens, dead or dying cells, immune complexes, and other antigens by phagocytosis, endocytosis and pinocytosis [188, 189].

Dendritic cells harboring antigens have a reduced capacity to uptake antigen during maturation [190]. Dendritic cells process endogenous, exogenous and lipid antigens and present them on their MHC class I, class II and CD1 molecules, respectively [191]. Dendritic cells are also able to cross-present exogenous antigen on their MHC class I molecules [192]. Maturation of DCs is accompanied with upregulation of

costimulatory molecules (CD80, CD86, CD40) and chemokine (CC) receptor (R) CCR7, enabling the DCs to home to the T cell area of lymphoid organs [193-195].

With the above features of DCs, they are able to efficiently prime naïve T cells by presenting peptide bound to MHC class I and class II molecules to CD4⁺ and CD8⁺ T cell receptors, respectively. T cells also require a second signal via costimulation of CD28 by CD86/80 expressed on DCs, without which anergy occurs [196]. CD4⁺ and CD8⁺ T cell responses induced by DCs could lead to antigen-specific adaptive immunity or tolerance.

T cell deletion and anergy have been shown to be the major factors that contribute to T cell tolerance induced by DCs. However, some subsets of DCs, presentation of autoantigens by DCs and the phenotype of DCs have been reported to be the key players in initiating such tolerance [197-200]. Early studies on the tolerogenicity of DCs revealed that APCs cross-presenting exogenous tissue-specific antigens through MHC class I lead to the deletion of autoreactive CD8⁺ T cells [198]. In addition, naïve CD4⁺ T cells were also found unresponsive in the presence of APCs bearing self-proteins [199]. Further, immature DCs expressing antigen have been demonstrated to dampen effector T cell responses and induce antigen-specific regulatory CD8⁺ T cells [200]. Interestingly, other studies have specifically implicated CD8 α ⁺ and CD8 α ⁻CD4⁺ DCs in mediating the induction of T cell tolerance to self-antigens [201]. Thus, while DCs in part play a crucial role in preventing autoimmunity, they may also be involved in tolerizing T cells to pathogen-associated antigens [201].

On the other hand, stimulation of naïve CD4⁺ and CD8⁺ T cells by DCs could result into the development of Th cells and CTLs, respectively, and in antigen-specific adaptive immune responses. Depending on the activated phenotype of DCs, they are able

to decide the type of Th responses induced, a Th1 or Th2-biased response. As mentioned before, DCs, particularly when activated by PAMPs of invading pathogens, secrete a variety of cytokines including IL-12 and IL-10. The cytokine microenvironment of Th cells created by DCs together with the subset of DCs polarizes their development towards a Th1 or Th2-biased subset. IL-12-producing DCs have been demonstrated to cause T cells to secrete large amounts of IFN- γ , which is a signature for a Th1-biased response. In contrast, IL-10-producing DCs drive T cells to secrete abundant IL-10, IL-5 and IL-4 indicating a Th2 phenotype. Furthermore, the Th1 and Th2 phenotypes have been associated with DCs of myeloid and lymphoid lineages, respectively [202]. In addition, DCs are also capable of secreting IL-15 for the sustenance of CD8⁺ T cell memory [203].

1.2.2 Heterogeneity of dendritic cells

In the last while, a significant number of distinct DC subtypes have been described. The phenotypes of the subpopulations of DCs are being distinguished to a large extent by their surface proteins, anatomic sites coupled with their distinct functions before infection and the downstream immune response they elicit after infection. According to Naik [204], DCs in their steady state can be subclassified into migratory DCs, lymphoid tissue-resident DCs and pDCs, while following an infection or inflammatory stimuli, they are described as inflammatory DCs.

Migratory DCs represent classical immature DCs, mainly located in peripheral tissues and sampling antigens in their environment. Upon receiving a maturation signal following the invasion of microbes or antigens from dead cells, they migrate to the draining lymphoid organs for antigen presentation. Epidermal Langerhans cells, dermal

DCs and interstitial DCs are mostly considered in this class of DCs. Migratory DCs are characterized by the level of expression of the surface proteins CD11c, CD11b, CD207, and the endocytic receptor CD205 [205, 206]. Not only do these DCs present antigens to T cells in the lymph nodes but they also have been shown to transfer antigen to lymph node-resident DCs for efficient CTL priming [206].

Lymphoid tissue-resident DCs are conventional DCs (cDCs) found in the lymphoid organs and spend their entire life living in these tissues. Most of these DCs have been demonstrated to be immature and may be involved in presenting antigen from infectious agents reaching the lymphoid organs [207]. DCs in this category express CD4 and CD8 receptors and are divided into two main groups: CD8 α^+ and CD8 α^- cDCs with CD8 α^- cDCs existing as CD4 $^+$ CD8 $^-$ and CD4 $^-$ CD8 $^-$ cDCs [208]. The CD8 α^+ and CD8 α^- cDCs have been shown to differ in the kind of immune responses they induce. CD8 α^+ DCs secrete large amounts of IL-12p70 and are capable of skewing the Th response to be Th1-biased while a Th2-type response is induced by CD8 α^- DCs [209]. On the contrary, another group reported that CD8 α^+ DCs initiated peripheral T cell tolerance [201]. Also, it has been reported that, although freshly isolated CD8 α^- DCs were able to better activate and induce expansion of naïve CD4 $^+$ and CD8 $^+$ T cells than CD8 α^+ DCs, both subtypes were equally potent in activating Th1-type responses after maturation. This suggests that the maturation state of these subsets was crucial in determining the type of Th response they direct [210].

Plasmacytoid DCs are type 1 IFN secreting cells and can be identified by the expression of DC antigen (CD11c) and B cell antigen (B220). Plasmacytoid DCs express low levels of the antigens CD11b, CD13, CD33 and are not efficient in phagocytosing or

endocytosing antigen but are capable of activating naïve T cells in allostimulation [211]. pDCs activated with CD40 ligand, unlike cDCs, induce significant amounts of Th2 cytokines and drive naïve CD4⁺ T cells to Th2-type cells [202]. In addition, due to the elevated IFN- α/β released by pDCs when stimulated by an inflammatory signal, they may be involved in the pathology associated with lupus erythematosus [212].

Inflammatory DCs are a new type of DCs that is absent in steady state. Following an infection or inflammation, DC precursors, mostly monocytes, in peripheral tissues, blood and bone marrow differentiate into inflammatory DCs (iDCs) and then migrate to lymphoid organs to induce antigen-specific T cell responses. Two subsets of inflammatory monocytes expressing high and low levels of the chemokine receptor CX₃CR1, traffic to noninflamed and inflamed tissues, respectively, and then differentiate into DCs [213]. It has been reported that inflammatory monocytes that require CCR2 to enter lymph nodes, developed into CD11c⁺CD11b^{hi}Gr-1⁺ iDCs that produced large amounts of IL-12p70 and induced Th1 immunity after viral infection or immunization [214]. Inflammatory DCs can also stimulate Th2 and Th17 responses [215, 216].

1.2.3. *In vitro* generation of dendritic cells

The pivotal role DCs play in immunity has won them attention as vectors for antigen delivery in vaccination studies. This would not have been possible if not for the robust culture systems available to generate DCs for studies and immunotherapeutic purposes. Dendritic cells represent about 0.42% of blood mononuclear cells of healthy individuals and also a minor cell population in mice [217, 218]. Recent advances in DC biology have made it much easier to expand progenitors of DCs (preDCs) to yield large

numbers of DCs *in vitro*. Presently, preDCs isolated from peripheral blood and organs, bone marrow and lymphoid organs supplemented with a variety of recombinant stem cell growth factors can be differentiated into DCs *ex vivo* according to one's needs. Classical DCs or cDCs that rise from preDCs are commonly generated with granulocyte macrophage-colony stimulating factor (GM-CSF) with or without IL-4 for up to at least 8 days depending on the origin of the preDCs. Not long ago, Brasel and colleagues [219] demonstrated that when they administered the ligand for the *fms*-like tyrosine kinase 3 (Flt3) receptor to mice, it induced the expansion of hematopoietic progenitors in the peripheral blood. Later, it was discovered that unlike GM-CSF/IL-4-generated DCs that are of a homogenous phenotype, Flt3 ligand (Flt3L) drives bone marrow cells into CD8⁺, CD8⁻ cDCs and pDCs [220]. The majority of the DCs differentiated in cell culture are relatively immature and are unable to prime T cells efficiently unless they are treated with the appropriate stimuli.

1.2.4. Loading dendritic cells with exogenous antigen

Dendritic cells generated *ex vivo* and used in immunotherapeutic studies must first be loaded with the antigen of interest. Antigens in the form of synthetic peptides or proteins, RNA, DNA or tumor-associated lysate are loaded into the DCs by either a viral or a non-viral method. Recombinant viruses bearing target genes can be used as vectors to transduce DCs. This approach, although efficient for loading antigens into DCs, has the disadvantage that some viral vectors may diminish DC maturation and function [221-223]. There also exists the possibility of viral antigens activating the host immune system to induce a response that may be detrimental to the host. Non-viral methods on

the other hand are much safer, since the host is not exposed to other viral genes beside that encoding the target antigen. Previously, the drawback with this method was the low transfection efficiency and viability following transfection of DCs with antigen by electroporation or liposome complexes [224]. With current improvements, high transfection efficiency can be achieved by transfecting DCs with mRNA of the target gene with minimal or no cytotoxicity when compared to using plasmid DNA or protein. In addition, the use of a nucleofector technology has been proven more efficient for transfer of mRNA into DCs than conventional electroporation and lipid-based transfection reagents [225, 226]. DCs can also be loaded with antigen *in vivo* via the endocytic receptor CD205, where the choice antigen is conjugated with monoclonal antibody to the indicated receptor [227].

1.2.5. Dendritic cell-based vaccine

Vaccine formulations consisting of the appropriate antigen of infectious agents with or without adjuvants in some cases activate the immune system successfully to clear an infection. Some pathogens, however, tend to silence the host immune responses to persist and as a result require a vaccination approach different from the above to combat them. Dendritic cell-based vaccination is one strategy that has shown significant promise in preclinical and clinical studies against difficult to treat diseases such as cancers, chronic human immunodeficiency virus (HIV) or HCV infections among others [226, 228, 229]. In a preclinical trial, where BM-DCs were loaded with HCV NS3/4A or NS5A mRNA, both T cell-mediated immune responses and protection against

recombinant vaccinia virus expressing HCV NS3/NS4/NS5A were induced in mice [175, 226]. However, despite the antigen-specific T cell responses elicited with DC-based vaccination, in a clinical trial against chronic HCV infection, there was no alteration in viral load, and the T cells responses were not prolonged [230]. In contrast, a sustained decrease in viral load was achieved in about 44% of chronic HIV-1 infected patients immunized with autologous DCs loaded with inactivated HIV-1 [229]. Some routes of administration have been found to be superior to others, and therefore may have to be exploited to attain robust immunity for clearance of HCV infection. Additionally, the subset of DCs could influence the outcome of protective immunity, as some DC subsets have been associated with Th2-biased responses and T cell tolerance as earlier discussed. Therefore, a consideration of these factors may help improve the efficacy of DC-based vaccines.

1.3 Toll-like Receptors

1.3.1 Dendritic cells and TLRs with their cognate ligands

Toll-like receptors belong to the family of PRRs, and as briefly described earlier, they are very essential for sensing invading microbial antigens during innate immunity via evolutionally conserved PAMPs. TLRs are expressed on several cell types such as DCs, macrophages, B cells, some T cells, fibroblasts and epithelial cells [231]. TLRs are present on the cell surface or endosomal compartments as type-1 transmembrane proteins with ectodomains comprised of leucine-rich-repeats for the recognition of their cognate ligands. They also possess an intracellular Toll-like interleukin 1 receptor (TIR) domain for downstream signaling upon initial activation from receptor-ligand complexes.

Thirteen TLRs have been identified, with 10 and 12 functional TLRs present in humans and mice, respectively. While TLR1 to TLR9 have been reported as conserved in these species, TLR10 is said to be non-functional in mice while TLR11, 12 and 13 are absent in the human genome [232]. Depending on the infecting microbe, TLRs are capable of detecting them by molecules in the form of nucleic acids, lipoproteins, lipids, proteins and also host derived antigens including heat shock protein, fibrinogen, among others [231]. Although DCs have been demonstrated to express all TLRs, the subtypes of DCs vary in their expression of TLRs. DC subsets ($CD4^+$ DC, $CD8\alpha^+$ DC, $CD4^-CD8^-$ DC and pDC) isolated from mouse spleen when examined, all expressed TLR1, 2, 4, 6, 8 and 9. However, TLR3, but not TLR5 and TLR7, preferentially occurred on $CD8\alpha^+$ DCs while TLR5 and TLR7 were expressed by pDCs and $CD4^-CD8^-$ DCs [233]. When the TLRs present on DCs or other cell types are engaged by their respective agonists, all TLRs except TLR3 signal through adaptor molecule MyD88 (myeloid differentiation primary response gene) to stimulate NF- κ B and MAPK (mitogen-activated protein kinases), which then induce inflammatory cytokine production. TLR3 signals through TRIF and activates IRF3 and NF- κ B to induce the secretion of inflammatory cytokines and type 1 IFN. TLR2 and TLR4, however, require the TIRAP/MAL (TIR-domain associated protein/MyD88-adaptor like) adaptor for recruitment to MyD88. In addition, TLR4 can also induce host defense responses through a TRIF-dependent pathway but requires the TRAM (TRIF-related adaptor molecule) for that to happen. An overview of TLR signaling has been provided by Kawai and Akira [232] and is shown in figure 1.2.

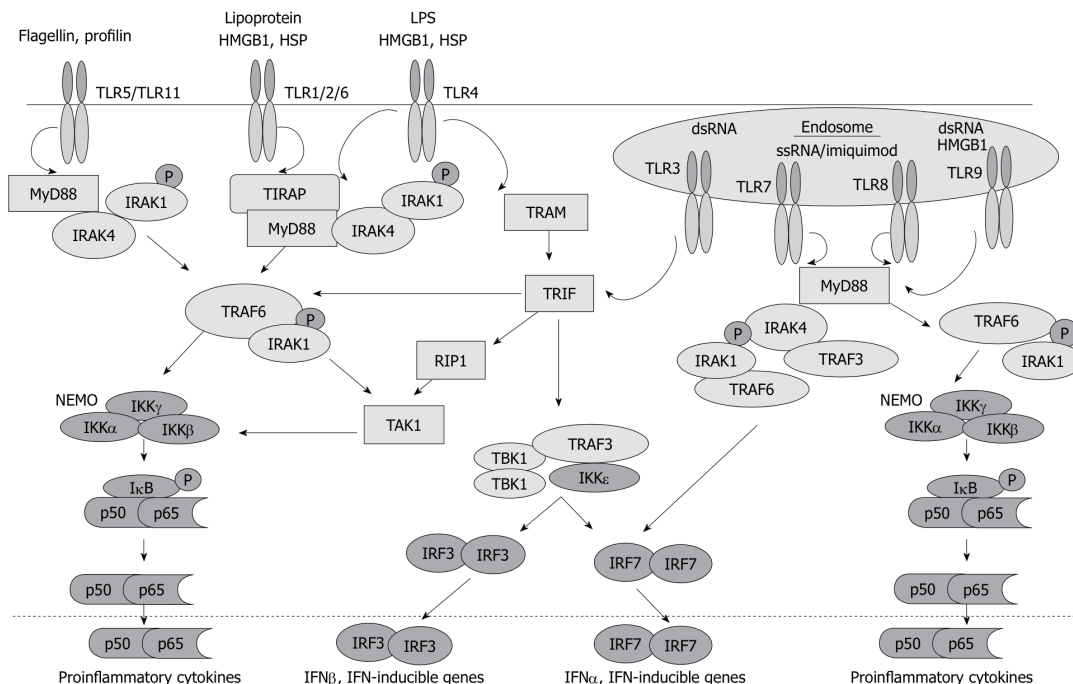


Figure 1.2. A summary of TLR signaling pathways indicating receptor ligands, adaptor molecules and the responses induced [234].

TLR-activated DCs show cytokine secretion and elevated expression of costimulatory molecules that is instrumental in the Th1/Th2 decision during acquired immunity. Thus, the immunostimulatory responses mediated by TLR ligands (TLR-Ls) have made them attractive adjuvants for most vaccine formulations.

1.3.2 Ajuvanticity of TLR ligands and synergy in vaccine formulations

When existing vaccine strategies fail to control a targeted infection, augmentation with suitable adjuvants to induce lasting protective immunity may be necessary. Utilization of single TLR-Ls as adjuvant has been effective in the stimulation of adaptive responses in the face of some infections but not all. Based on recent studies it is now

known that combinations of some TLR-Ls cooperate to amplify immune responses [235, 236]. One study indicated that when either TLR4 or TLR9 ligand was used as adjuvant together with Leishmania antigen in a vaccine formulation, high multifunctional T cells responses were induced in the absence of infection but not during active infection. However, when the combination of those ligands was used, reduced parasite load was achieved [237]. DCs activated with combined TLR agonists that trigger both MyD88- and TRIF-dependent pathways synergistically enhance costimulatory molecules, activation of CD4⁺ and CD8⁺ T cells, Th1 promoting cytokines including IL-12, IFN- γ , and subsequently induce stronger Th1-biased responses *ex vivo* and *in vivo* when compared to the effect from a single TLR-L [236, 238]. Further, the triple combination of TLR2/6, TLR3 and TLR9 agonists prolonged DC survival through amplification of IL-15 release and improved the magnitude and quality of T cell responses induced [239]. In contrast, concurrent stimulation of DCs with a combination of TLR-Ls whose TLR signal through the same pathways did not result in a synergistic effect [238].

2.0 HYPOTHESIS AND OBJECTIVES

In a previous preclinical study, superior transfection efficiency with DCs loaded with HCV NS5A mRNA and corresponding protective immunity was demonstrated when compared with DCs loaded with HCV NS5A plasmid or protein [226]. Complete protection was not however, achieved in this study, probably because the DCs used were not optimally activated. Thus, the overall goal was to improve the efficacy of this vaccination strategy.

In this study I proposed that adoptive transfer of DCs loaded with HCV NS3/4A and/or NS5A mRNA and treated with TLR agonist(s) *ex vivo* would induce potent HCV-specific responses *in vivo*. To test this hypothesis, the following specific objectives were carried out.

1. Test the impact of single or combined TLR agonist treatment on the phenotypes of bone marrow-derived DCs (BMDCs).
2. Evaluate the functional characteristics of the TLR agonist(s)-treated DCs transfected with HCV NS3/4A, NS5A mRNA or combinations of NS3/4A and NS5A *in vitro*.
3. Assess cellular immune responses elicited in mice vaccinated with DCs carrying HCV antigen(s) and activated with single or combined TLR-Ls.
4. Evaluate protection of mice immunized with DCs loaded with HCV mRNA(s) and challenged with recombinant vaccinia virus encoding HCV NS3/NS4/NS5 genes.
5. Determine adaptive responses elicited following vaccination with HCV recombinant NS3 or NS5A protein co-formulated with a triple adjuvant containing a TLR-L.

3.0 MATERIALS AND METHODS

3.1 Generation of bone marrow-derived DCs

Murine BMDCs were generated as previously described by Inaba *et al.* [240] with some modifications. Briefly, bone marrow cells were flushed out of femurs and tibia of naive Balb/c mice (H-2^d) purchased from Charles Rivers Laboratories. Bone marrow cells were depleted of red blood cells (RBCs) using 5 milliliters (ml) of ammonium chloride lysis buffer (0.14 M NH₄Cl in 17 mM Tris pH 7.2) for 5 minutes (min). This was followed by addition of 30 ml of phosphate buffer saline (PBS), pH 7.2 (Invitrogen). Cells were passed through 70 µm cell strainers (BD Biosciences) to remove any pieces of bones and debris. The cells were then washed twice with PBS, pH 7.2 (Invitrogen) and resuspended in complete RPMI, which consisted of RPMI 1640 medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (PAA Laboratories, Inc.), 10 mM HEPES buffer (Invitrogen), 0.1 mM non-essential amino acids (Invitrogen), 2 mM L-glutamine (Invitrogen), 1 mM sodium pyruvate (Invitrogen), 50 µM β-mercaptoethanol (Sigma-Aldrich) and 50 µg/ml of gentamicin (Invitrogen). Further, GM-CSF (R&D Systems) was added at a final concentration of 10 ng/ml. Bone marrow cells were plated at a concentration of approximately 1×10^6 cells/ml in 6-well tissue culture plates (Corning Incorporated) and incubated at 37°C and 5% CO₂. On day 2 of culture, non-adherent cells were removed after rocking the culture plates 2-4 times, without dislodging loosely adherent clusters of cells formed. Fresh warm complete RPMI medium containing GM-CSF was added to the culture plates and incubated for another 2 days. On days 4 and 6, 50% of the culture medium was replenished with fresh warm medium plus GM-CSF. Cells were collected for different purposes on day 8.

Alternatively, bone marrow cells were cultured in the presence of 100 ng/ml Flt3L (Peprotech) for 8 days [241]. On day 4, 50% of the culture medium was replaced with fresh warm complete RPMI medium containing Flt3L, and cells were collected on day 8.

3.2 *In vitro* maturation of DCs

Day 8 BMDCs generated with either GM-CSF (GM-DCs) or Flt3L (FL-DCs) were collected, washed in PBS pH 7.2 (Invitrogen), resuspended in fresh complete RPMI medium and plated at a concentration of 1×10^6 cells/ml in a 24-well tissue culture plate (Corning Incorporated). GM-DCs or FL-DCs were left untreated or treated with 1 µg/ml ultrapure lipopolysaccharide (LPS) (Invivogen), 500 ng/ml R848 (imidazoquinoline) (Invivogen), 3 µg/ml CpG ODN 1826 (Merial Duluth), 25 µg/ml polyinosinic:polycytidylic acid (Poly I:C) (Invivogen) or a combination of 25 µg/ml Poly I:C and 3 µg/ml CpG ODN 1826 for 20 hours (h) or the indicated time prior to harvest. Doses used were determined to be optimal after titration.

3.3 Flow cytometric analysis of surface molecule expression

GM-DCs and FL-DCs left untreated or treated with TLR-L(s) were collected on day 9, washed twice with PBS, and resuspended in FACOLA (10 mM PBS pH 7.2, 0.2% gelatin, 0.03% sodium azide) to stain cell surface proteins. Two hundred thousand cells in 50 µl volumes were added to 50 µl of FACOLA in a round-bottom 96-well tissue culture plate (Nalgen Nunc International). Cells were incubated on ice in the dark for 30-45 min with the following directly conjugated murine monoclonal antibodies obtained from BD

Pharmingen: I-A^d (10 µg/ml, fluorescein isothiocynnate (FITC)), IgG₃κ (10 µg/ml, FITC), CD40 (10 µg/ml, FITC), CD86 (10 µg/ml, FITC), IgG_{2a}κ (10 µg/ml, FITC), B220 (10 µg/ml, FITC), CD11c (4 µg/ml, phycoerythrin (PE)), IgG1_λ (4 µg/ml, PE), CD11b (4 µg/ml, allophycocyanin (APC)), IgG_{2b}κ (4 µg/ml, APC). Cells were then washed twice with FACOLA, resuspended in 200 µl of 2% formaldehyde and kept at 4 °C until ready to be analyzed. Cells were analyzed by collecting 10,000 events per sample using a FACSCalibur flow cytometer with CellQuest software (BD Biosciences).

3.4 Isolation of splenocytes

Spleens were isolated from Balb/c or C57BL mice into 15 ml tubes with 5 ml wash medium consisting of minimum essential medium (MEM) (Sigma Aldrich), 10 mM HEPES (Invitrogen), 50 µg/ml gentamicin (Invitrogen) per spleen on ice. Spleens in the wash medium were poured into 100 µm cell strainers (BD Biosciences) placed in a petri dish (VWR); excess fat on the spleens was removed and the spleens were cut into small pieces with a scissors. With a plunger of a glass syringe (Popper and Sons Inc.), cells were gently teased out of the spleens through the cell strainers. The cell strainers were washed with 5 ml of wash medium and cells were passed through the strainers 4 times to remove any clumps. Cells were centrifuged at 311 x g and 4 °C for 10 min. Spleen cells were depleted of RBCs by resuspending the cell pellet in 1 ml ammonium chloride lysis buffer (0.14 M NH₄Cl in 17 mM Tris pH 7.2) for 30 seconds (sec) at room temperature. Subsequently, cells were washed twice with 10 ml wash medium and resuspended in complete RPMI medium or complete AIM-V made of AMIV medium (Invitrogen) supplement with 10 mM HEPES (Invitrogen), 0.1 mM non essential amino acids

(Invitrogen) 1 mM sodium pyruvate (Invitrogen) and 50 μ M 2-mercaptoethanol (Sigma Aldrich) after the last wash. Splenocytes were counted using the Coulter counter (Beckman Coulter Inc.).

3.5 T cell isolation by magnetic separation with LS columns

Splenocytes from Balb/c or C57BL mice were enriched for CD4⁺ and CD8⁺ T cells by using mouse CD4 and CD8 α microbeads (Miltenyi, Biotec) according to the manufacturer's protocol. Splenocytes were resuspended in MACS buffer (PBS, pH 7.2 containing 2 mM EDTA, 0.5% bovine serum albumin) and labeled with 10 μ l CD4 and CD8 α microbeads per 10⁷ cells. After 20 min of incubation on ice, MACS buffer was added to the cells, followed by centrifugation at 300 x g for 10 min. Cell pellets were resuspended in 0.5 ml of MACS buffer. Subsequently, LS columns (Miltenyi Biotec), placed in a magnetic field of midi MACS separators (Miltenyi Biotec), were rinsed with 3 ml of MACS buffer, and the cells were applied to the columns. Unlabeled cells were allowed to pass through the columns, after which the columns were washed 3 times with 3 ml MACS buffer. LS columns were removed from the magnetic field, and CD4⁺ and CD8⁺ T cells were flushed out into 15 ml tubes with 5 ml of MACS buffer. CD4⁺ and CD8⁺ T cells were pooled together and centrifuged at 300 x g for 10 min, and the cell pellets were resuspended in complete RPMI medium at a concentration of 2×10^6 cells/ml.

3.6. Mixed lymphocyte reaction

Bone marrow-derived DCs from Balb/c mice resuspended in complete RPMI medium at a concentration of 2×10^5 cells/ml were gamma-irradiated at 5000 rads. Bone marrow-derived DCs were then plated in triplicate wells of round-bottom 96-well tissue culture plates at 4-fold dilutions. Pooled CD4⁺ and CD8⁺ T cells from Balb/c or C57BL mice at a concentration of 2×10^6 cells/ml were added to the BMDCs to obtain the following BMDC to T cell ratios: 1:10, 1:40, 1:160 and 1:640, unless otherwise indicated. The BMDC-T cell cocultures were incubated at 37°C and 5% CO₂ for 5 days. The cultures were pulsed with 0.4 µCi/well of methyl ³[H] thymidine (Amersham Pharmacia Biotech) during the last 18 h of incubation. Cells were harvested with a Filter Mate Harvester (Packard Bioscience Company) and the radioactivity was determined by a TopCount NXTTM Microplate scintillation and luminescence counter (Packard Bioscience Company). Data obtained were expressed in counts per minutes (CPM).

3.7 *In vitro* transcription (IVT) of mRNA

pGEM4Z NS5A or pGEM4Z NS3/4A were constructed by Dr. Yu by replacing the eGFP plasmid in pGEM4Z 5'UT-eGFP-3'UT-A64 (kindly provided by Dr E. Gilboa, Duke University), with HCV NS5A or NS3/NS4A. The pGEM4Z eGFP, plasmid is flanked by a 5' and 3' untranslated regions and a poly A tail. pGEM4Z eGFP, pGEM4Z NS3/NS4A and pGEM4Z NS5A were linearized with SpeI restriction enzyme (New English Biolabs), followed by *in vitro* transcription using a T7 mMessage mMachine Kit from Ambion. The IVT reaction mix was kept at 37°C for 2 h. Turbo DNase 1 (Ambion) was then used to clean up template plasmid at 37°C for 15 min. The mRNA transcribed

was recovered by lithium chloride (Ambion) precipitation. The quantity and integrity of the mRNA were determined using a Nanodrop-spectrophotometer (Nanodrop Technologies) and agarose gel electrophoresis.

3.8 Transfection of DCs

Bone marrow-derived DCs collected on day 8 were washed twice with PBS, pH 7.2 (Invitrogen), and the cell number was determined. Cells were resuspended in Opti-MEM (Invitrogen) for electroporation at a concentration of 20×10^6 cells/ml or in nucleofector reagent (Lonza) specific for mouse DCs for nucleofection at a final concentration of 40×10^6 cells/ml. Next, 200 μ l and 100 μ l of cell suspension were transferred into 0.4-cm and 0.1-cm sterile cuvettes for electroporation and nucleofection respectively. Cells were mixed gently with 30 μ g of mRNA and electroporated using the Gene Pulser Xcell (Bio-rad) with the indicated parameters (300V or 350V, 100 - 300 μ F and 200 Ω) or nucleofected using the Amaxa nucleofector (Lonza) with programs K2, U2, X1 and Y1. Shortly after transfection, cells were resuspended in warm complete RPMI supplemented with 100 ng/ml Flt3L (Peprotech) or 10 ng/ml of GM-CSF (R&D Systems) and incubated at 37°C until further use.

3.9 Immunocytochemistry

Since the GM-DCs are more adherent than the FL-DCs, FL-DCs transfected with HCV NS3/NS4A and/or NS5A mRNA were spun onto glass slides, while the GM-DCs loaded with HCV mRNA(s) were plated at 2×10^5 cells per well into 8-well LAB-TEK chamber slides (Nalge Nunc International). Cells were fixed for 20 min with 4%

paraformaldehyde (Sigma Aldrich) in PBS and permeabilised with 0.5% Triton X-100 (Fisher) in PBS for 10 min. Cells were then washed once with PBS and blocked with 1% horse serum in PBS for 20 min or overnight. The cells were incubated with monoclonal antibodies (mAb) specific for HCV NS3, NS4A and/or NS5A (Meridian Life Science) diluted 1:200 in 1% horse serum/PBS (diluent) for 1.5 h, and then washed 3 times for 5 min. Subsequently, cells were incubated with biotinylated horse anti-mouse IgG (H+L) (Vector Laboratories) at a 1:2000 dilution for 1.5 h and further incubated with ABC reagent (Vector Laboratories) for 45 min. Finally, cells were incubated with diaminobenzidine (DAB) peroxidase substrate (Vector Laboratories) until the desired stain intensity had developed. Slides were washed in distilled water, dried and observed under a Zeiss Axiovert microscope (Carl Zeiss).

3.10 Cytokine ELISA

Supernatants from BMDC cultures treated with TLR-L(s) or left untreated were used to quantify IL-12p70, IL-10, IFN- γ and IL-5 secreted by the DCs. Also, supernatants obtained from BMDC-T cell cocultures were used to quantify IFN- γ and IL-5 secreted by the T cells. Supernatants were kept at -20°C until ready to use. These cytokines were quantified with the respective Ready-Set Go! ELISA kits (eBioscience) by following the manufacturer's procedures.

3.11 Production of HCV recombinant proteins

The HCV NS3 and NS5A genes were amplified from the pHCVrep1b1 plasmid and cloned into the expression vector, pRSET (Invitrogen) by Dr. Yu. Subsequently, the

HCV NS3 and NS5A genes were cut out of the pRSET vector and cloned into the pET30a expression vector (Novagen). *Escherichia coli* (*E. coli*) BL21 (DE3) cells were then transformed with pET30a-NS3 or pET30a-NS5A. Expression of HCV recombinant (r) NS3 or NS5A protein was induced with isopropyl β -1-D-thiogalactopyranoside (Sigma Aldrich) for 3 h as inclusion bodies. *E. coli* BL21 cells were spun and the cell pellets were resuspended in PBS containing protease inhibitor cocktail (Sigma Aldrich) and sonicated 15 times at a 20 sec burst until the cells were completely lysed. Cell pellets obtained after centrifugation were solubilised in lysis buffer (table 3.1) overnight. Supernatants from solubilized proteins were incubated with 1 ml His60 resin (Clontech) for 1 h. Following two washes of the resin with 10 resin volumes of resin with wash buffer 1 and 2 (table 3.1), recombinant proteins were eluted 10 times with 1 ml of elution buffer each time. Recombinant proteins were dialyzed against different concentrations of urea, resolved on 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, and further identified by Western blotting (Fig. 3.1). Bands below the indicated recombinant proteins are likely degraded products of the proteins. Protein concentrations were determined by Bradford assay (Biorad). Endotoxins present in the recombinant protein preparations were removed with a proteospin endotoxin removal kit (Norgen Biotek). Endotoxin levels were then determined with the Limulus Amoebocyte Lysate QLC (Lonza), to be below 0.1EU/ μ g.

Table 3.1. Buffers used in protein purification

Buffer	Constituents
Lysis Buffer pH 7.4	6M GuHCL 50mM NaH ₂ PO ₄ 300mM NaCl 20mM Imidazole (Sigma-Aldrich) 2% NP-40 (Sigma-Aldrich)
Wash Buffer 1 pH 7.4	8M Urea (EMD) 50mM NaH ₂ PO ₄ 300mM NaCl 40mM Imidazole (Sigma-Aldrich) 2% NP-40 (Sigma-Aldrich) 0.1% Triton X-114 (Sigma-Aldrich)
Wash Buffer 2 pH 7.4	8M Urea (EMD) 50mM NaH ₂ PO ₄ 300mM NaCl 60mM Imidazole (Sigma-Aldrich) 2% NP-40 (Sigma-Aldrich) 0.1% Triton X-114 (Sigma-Aldrich)
Elution Buffer pH 7.4	8M Urea (EMD) 50mM NaH ₂ PO ₄ 300mM NaCl 300mM Imidazole (Sigma-Aldrich) 2% NP-40 (Sigma-Aldrich)

The other products were obtained from Bio basic Canada Inc.

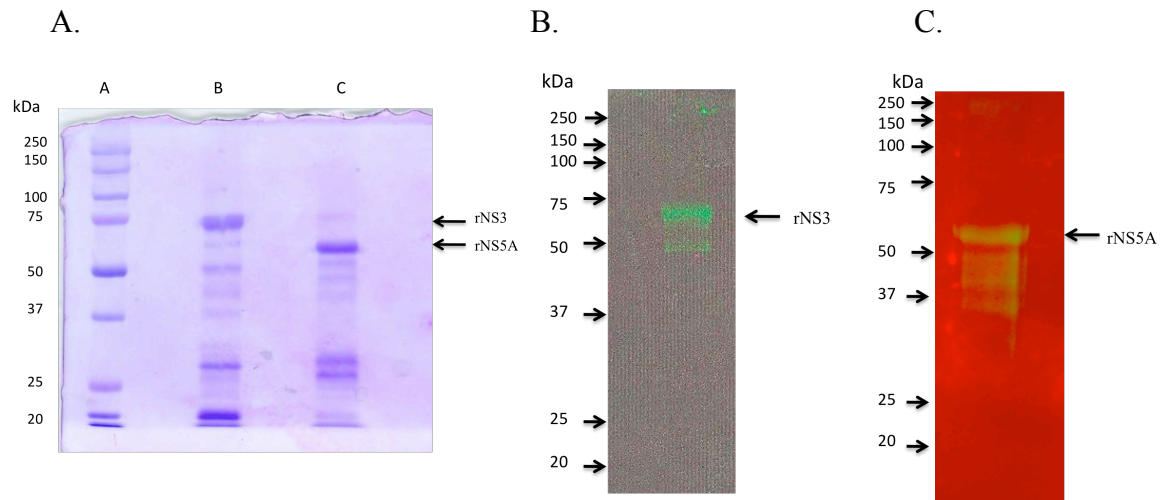


Figure 3.1. Identification of purified HCV recombinant proteins. HCV rNS3 and rNS5A proteins were identified by SDS-PAGE (A) and Western blotting (B and C). Lane A (molecular weight markers), lane B (purified rNS3) and lane C (purified NS5A).

3.12 Cell line, propagation and purification of recombinant virus.

African green monkey kidney epithelial (BSC-1) cells were grown in 18 ml of MEM (Sigma-Aldrich) supplemented with 10% FBS (PAA Laboratories, Inc.), 10 mM Hepes (Invitrogen), 0.1 mM non-essential amino acids (Invitrogen) and 50 µg/ml of gentamicin (Invitrogen). Cells were cultured in a T150 flask (BD Biosciences) at 37°C in 5% CO₂. BSC-1 cells were allowed to grow until about 100% confluency was achieved. The cell culture medium was removed and replaced with 5 ml of trypsin-EDTA (Invitrogen) made in versene to trypsinize the cells. After cells were dislodged from the flask, 8 ml of cell culture medium was added to cells to inactivate the trypsin-EDTA. Cells were centrifuged in a 15 ml tube at 216 x g for 10 min and split at a ratio of 1:3 every third day.

For the propagation of recombinant vaccinia virus (rVV) expressing HCV NS3/NS4/NS5 (a gift from Sanofi Pasteur MSD), about 100% confluent BSC-1 cell monolayers were infected with a multiplicity of infection (MOI) of 0.1 of the recombinant virus in 5 ml FBS-free culture medium. After 2 h, 10 ml of culture medium containing 4.5% FBS (PAA Laboratories, Inc.) was added to the cells and the infection was allowed to continue for 48 h. Virus-infected cells were kept at -80°C until used. To purify the rVV, flasks containing infected cells were thawed, and attached cells were removed with a cell scraper (Corning Incorporated). The virus suspension was transferred into 50 ml Falcon tubes (BD Biosciences), vortexed and centrifuged at 1800 x g for 5 min. Cell pellets were resuspended in 10 ml of Tris-HCl (Bio basic Canada Inc.), pH 9, vortexed and centrifuged at 1800 x g for 5 min. The supernatants containing the virus suspensions were pooled and laid over a 36% sucrose cushion (Bio basic Canada Inc.) in

ultracentrifugation tubes (Beckman Coulter Inc.), which were then spun for 1 h at 32,000 x g and 4°C. Subsequently, virus pellets were resuspended in 1 mM Tris-HCl, pH 9.0, and stored at -80°C. Recombinant virus was quantified by plaque assay.

3.13 Immunization and virus challenge of mice

Groups of naïve female Balb/c mice (Charles Rivers Laboratories) were vaccinated with PBS, or with 5×10^5 untreated or activated mRNA-transfected DCs unless otherwise indicated. Mice were immunized two times with a two-week interval between the first and second vaccination. In recombinant protein vaccine studies, each mouse was vaccinated with 4 µg rNS3 or rNS5A protein co-formulated with 10 µg poly [di(sodium carboxylatoethylphenoxy)-polyphosphazene] (PCEP) (Idaho National Laboratory), 10 µg CpG ODN10101 (TCGTCGTTTTCGCGCGCGCGCCG) (Pfizer) and 20 µg IDR1002 (VQRWLIVWRIRK) (Genscript) [242] in PBS, pH 7.4 (Invitrogen). Mice were immunized twice with a three-week interval.

Regardless of the type of vaccine administered, ten days after the last vaccination half of the mice in each group were euthanized, and spleens from these mice were removed for splenocyte isolation. To detect specific immune responses, splenocytes were used for enzyme-linked immunospot (ELISPOT) assays and intracellular cytokine staining. The remaining mice in each group were challenged intraperitoneally (i.p) with 5×10^6 pfu of rVV encoding HCV NS3/NS4/NS5 antigens. Five days later, the mice were euthanized, and the ovaries collected to measure virus replication.

3.14 IFN- γ and IL-5 ELISPOT assays

Ninety-six-well multiscreen-HA ELISPOT plates (Millipore) were coated one day before splenocyte isolation from immunized mice with 100 μ l per well of anti-mouse IFN- γ and IL-5 mAb (BD PharMingen) diluted in sterile coating buffer (125 mM Na₂CO₃ with 375 mM NaHCO₃, pH 9.6) to a final concentration of 2 μ g/ml. After keeping the plates overnight at 4°C, the plates were washed 4 times with PBS, pH 7.2 (Invitrogen), and blocked with 1% bovine serum albumin (BSA) (Sigma-Aldrich) in PBS (Invitrogen) (BSA/PBS) for 2 h at 37°C in a 5% CO₂ incubator. The blocking buffer was discarded, and 1×10^6 splenocytes from each mouse were added in 100 μ l to triplicate wells of the plates in the presence of medium or 1 μ g/ml final concentration of HCV rNS3 and/or rNS5A protein(s) or concanavalin A (ConA). After 20 h of incubation, the cultures were discarded and plates were washed twice with PBST (PBS with 0.05% Tween (Sigma-Aldrich)) followed by cell lysis with double distilled water (ddH₂O) for 5 min at room temperature. Plates were further washed 3 times with PBST and 2 times with ddH₂O. Biotinylated anti-mouse IFN- γ and IL-5 mAb (BD PharMingen) diluted to a concentration of 2 μ g/ml in 1% BSA/PBS were added to each well in a 100 μ l volume for 1-2 h at room temperature. Next, the plates were washed as described above, and 100 μ l of alkaline phosphatase (AP)-conjugated streptavidin (Jackson ImmunoResearch Laboratories) diluted 1:1000 in 1% BSA/PBS was added to each well for 1-2 h. To develop spots, plates were washed once again, and 100 μ l of 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium substrate (Sigma-Aldrich) dissolved in water was added to each well. Ten minutes after the spots were developed, plates were washed with ddH₂O and dried.

3.15 Intracellular cytokine staining

In round-bottom 96-well tissue culture plates, of 1×10^6 splenocytes in 100 μ l were stimulated with a MOI of 0.1 of rVV expressing HCV NS3/NS4/NS5, as described by Speller *et al.* [243], but with some modifications. Stimulated cells were incubated overnight at 37°C and 5% CO₂, followed by the addition of Golgiplug (BD Biosciences) at 1 μ l/ml of culture medium for another 10 h. Cells were then washed with FACOLA, and double stained with directly conjugated mouse mAb (all obtained from BD Biosciences) specific for CD3-PE (10 μ g/ml) and CD4-FITC (5 μ g/ml) or CD8-FITC (5 μ g/ml) on ice in the dark for 30 min. Subsequently, cells were washed with FACOLA and then permeabilised with cytofix/cytoperm solution (BD Biosciences) for 20-30 min. After two washes with perm/wash buffer (BD Biosciences), cells were stained with 2 μ g/ml of anti-mouse IFN- γ -APC or isotype control-APC mAb (BD Biosciences) for 30 min on ice in the dark. The cells were again washed with the perm/wash buffer (BD Biosciences), resuspended in 200 μ l PBS and kept at 4°C until ready to be analyzed. Cells were analyzed by collecting 15,000 events per sample using a FACSCalibur flow cytometer with CellQuest software (BD Biosciences).

3.16 Serum antibody ELISA

Immunlon II plates (Thermo Scientific) with 96 wells were coated with 100 μ l of rNS3 or rNS5A diluted in coating buffer (125 mM Na₂CO₃ with 375 mM NaHCO₃, pH 9.6) to a final concentration of 400 ng/ml. The plates were wrapped in plastic and kept at 4°C overnight. Plates were washed as described in the ELISPOT assay and blocked for 1 h with PBS containing 5% gelatin, which was used as the diluent in the subsequent steps

of the assay. Sera from immunized animals serially diluted in 5% gelatin/PBS (starting with 1:40 for IgG and 1:100 for both IgG1 and IgG2a) were added to the plates. The plates were wrapped with plastic and incubated at 4°C overnight. Alkaline phosphatase-conjugated goat anti-mouse IgG (Kirkegaard & Perry Laboratories), diluted to 1:5000, or biotin-conjugated goat anti-mouse IgG1 or IgG2a (Southern Biotech), diluted 1:10,000, was added to the plates and incubated at room temperature for 1 h after washing. Plates were washed and AP-conjugated streptavidin (Jackson ImmunoResearch Laboratories) diluted 1:20,000 was added to the IgG1 or IgG2a, but not the IgG plates, for 45 min at room temperature. The plates were washed again and developed with p-nitrophenyl phosphate (Sigma-Aldrich) diluted 1:100 in DE buffer, pH 9.8 (1 M diethanolamine with 0.5 mM MgCl₂). The absorbance was read at 405 nm with a reference reading at 490 nm. Antibody titers were calculated as the reciprocal of the highest dilution resulting in a value of two standard deviations above the average value of the negative control sera.

3.17 Protection and plaque assay

One day before the collection of ovaries, 2×10^4 BSC-1 cells in 100 µl per well were plated in flat-bottom 96-well tissue culture plates (Nalge Nunc International) and incubated at 37°C and 5% CO₂. Ovaries from mice challenged with rVV expressing HCV NS3/NS4/NS5 were collected in 2 ml eppendorf tubes containing 200 µl of MEM supplemented with 10 mM HEPES (Invitrogen), 0.1 mM non-essential amino acids (Invitrogen) and 50 µg/ml of gentamicin. Each pair of ovaries was homogenized together with a PRO 200 homogenizer (PRO Scientific). Homogenates were subjected to 3 cycles of quick freeze-thaw. Homogenates were serially diluted 10 fold in triplicate wells of flat-

bottom 96-well plates. BSC-1 cells were incubated with 100 µl of the diluted homogenates for 1.5 h at 37°C and 5% CO₂. After removal of the diluted homogenates, 200 µl of MEM supplemented with 2% FBS (PAA Laboratories Inc), 10 mM HEPES (Invitrogen), 0.1 mM non-essential amino acids (Invitrogen) and 50 µg/ml gentamicin (Invitrogen) was added to each well, and cells were further incubated for 48 h. The amount of rVV expressing HCV NS3/NS4/NS5 in the ovaries of the challenged mice was determined after the cells were stained with 0.5% w/v of crystal violet.

3.18 Statistical analysis

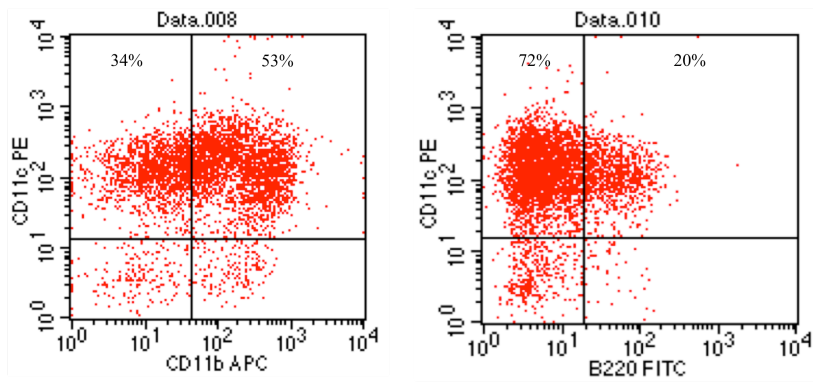
Statistical Analyses were performed using Graphpad Prism Version 6 software for Mac. Analysis of variance (ANOVA) with Bonferroni multiple comparison test was used to determine significant differences between data sets. P values < 0.05 were considered statistically significant

4.0 RESULTS

4.1 Phenotypic characteristics of FL-DCs and GM-DCs

To assess the phenotype of the FL-DCs and GM-DCs, we characterized both DCs based on the expression of the surface proteins CD11c, CD11b and B220. FL-DCs contained a mixed population of CD11c⁺CD11b⁺ and CD11c⁺B220⁺ cells, which represent conventional and plasmacytoid DCs, respectively (Fig 4.1A). The majority of the CD11c⁺ DCs generated with GM-CSF were CD11b⁺, and only 0.5% of the cells were CD11c⁺B220⁺ (Fig. 4.1B). The GM-CSF DCs thus represent mostly conventional DCs as previously described [220, 244, 245]. The phenotype and morphology of FL-DCs and GM-DCs treated with Poly I:C (TLR3-L), LPS (TLR4-L), R848 (TLR7-L) or CpG ODN (TLR9-L) were examined. In the absence of a maturation stimulus, FL-DCs looked small and lymphoid-sized relative to GM-DCs. FL-DCs and GM-DCs appeared round to irregular in shape and showed little or no dendrites (Fig. 4.2A and 4.2C). The activated phenotype of both FL-DCs and GM-DCs displayed long dendrites (Fig. 4.2B and 4.2D) and showed elevated expression of the costimulatory molecules CD86 and CD40, as well as MHC class II, compared to their counterparts not treated with TLR-L (Fig. 4.3A and 4.3B).

A.



B.

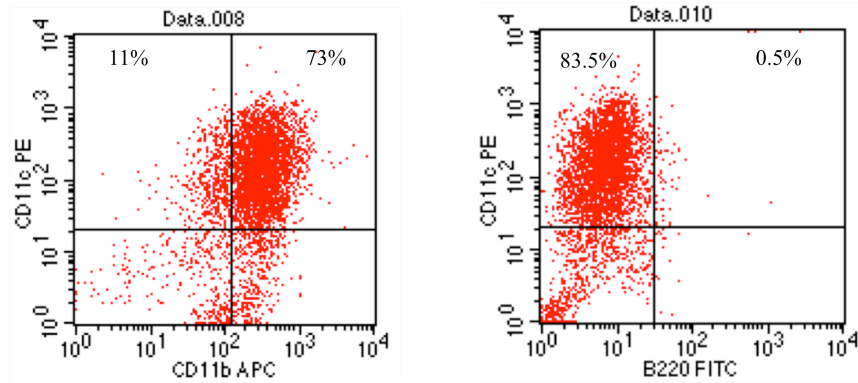
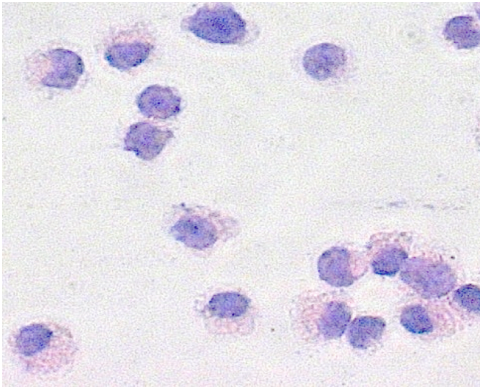
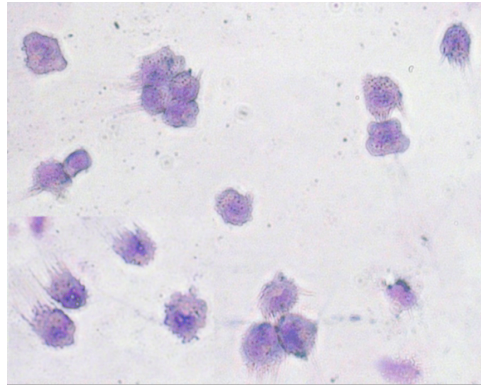


Figure 4.1. Differential expression of surface proteins by FL-DCs and GM-DCs. Bone marrow cells from Balb/c mice were cultured in the presence of (A) 100 ng/ml of recombinant mouse Flt3L or (B) 10 ng/ml of recombinant mouse GM-CSF for 8 days. Cells were collected, washed and stained for CD11c, CD11b and B220 markers. Expression of surface markers was determined by flow cytometry.

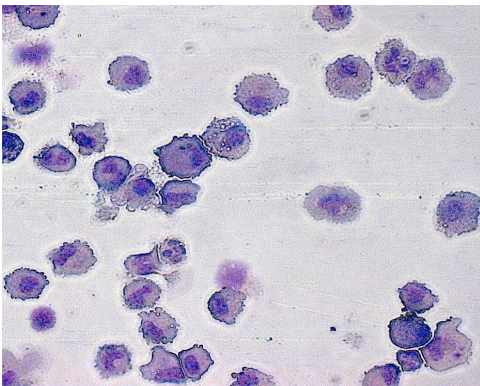
A.



B.



C.



D.

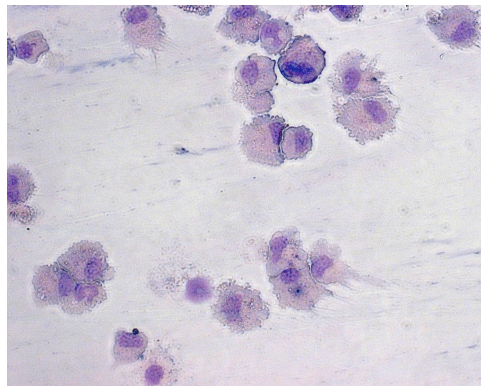


Figure 4.2. Morphology of FL-DCs and GM-DCs. BMDCs generated with (A and B) Flt3L and (C and D) GM-CSF were left (A and C) untreated or (B and D) treated with CpG ODN for 18-20 h. Cells were spun onto glass slides and stained with Giemsa stain.

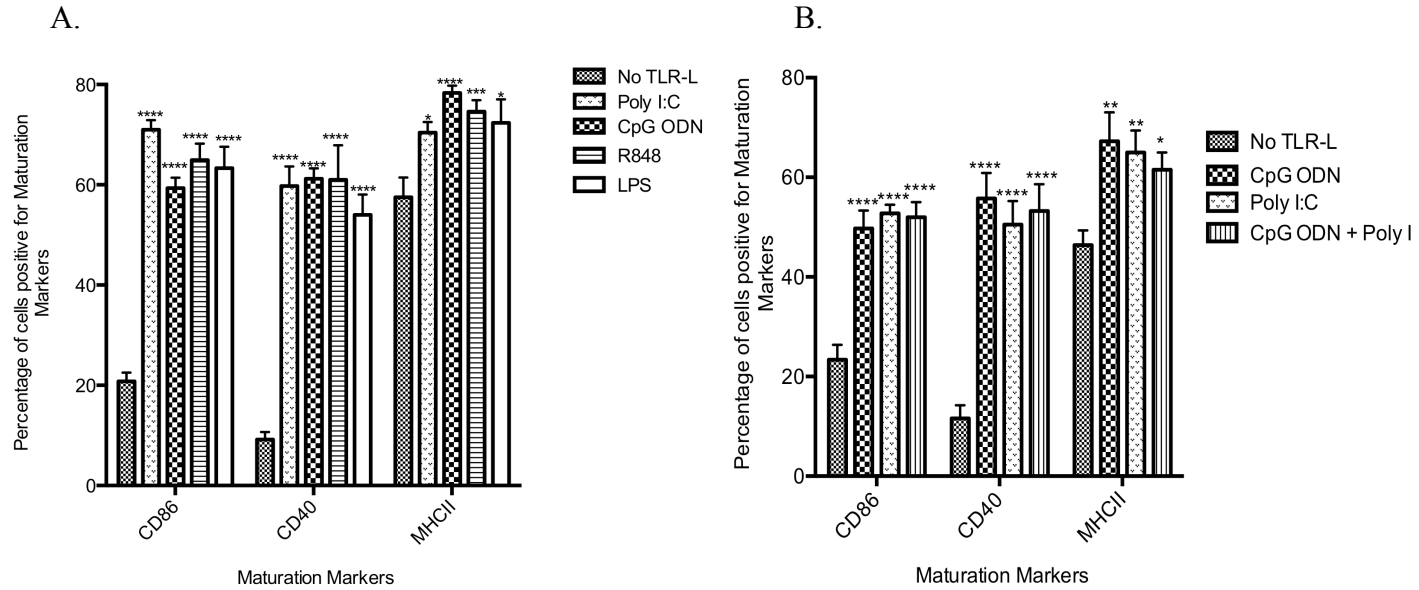


Figure 4.3. TLR ligand-treated FL-DCs and GM-DCs have elevated expression of maturation markers. Day 8 (A) FL-DCs and (B) GM-DCs were left untreated or treated with CpG ODN (3 $\mu\text{g/ml}$), R848 (500 ng/ml), Poly I:C (25 $\mu\text{g/ml}$) or LPS (1 $\mu\text{g/ml}$) for 20 h. Cell surface proteins were stained and the expression of the indicated maturation markers was assessed by flow cytometry. Data represent mean with SEM of 4 (A) and 3 (B) independent experiments. **** $P<0.0001$, *** $P<0.001$, ** $P<0.01$, * $P<0.05$: significance of difference between DCs treated with Poly I:C, CpG ODN R848 or CpG ODN plus Poly I:C and DCs not treated with TLR-L.

4.2 Influence of TLR agonist treatment on FL-DCs and GM-DCs

4.2.1 Combination of CpG ODN and Poly I:C augments IL-12p70 production.

Cytokine release by FL-DCs in response to treatment with the different TLR agonists was assessed. All TLR-L treatments caused FL-DCs to produce both IL-12p70 and IL-10, while no cytokines were secreted in the absence of TLR ligation of FL-DCs. CpG ODN-stimulated FL-DCs secreted equal amounts of IL-12p70 and IL-10. Activation with Poly I:C, LPS or R848, however, induced the production of more IL-10 than IL-12p70 (Fig. 4.4A).

Furthermore, the effect of combinations of TLR-Ls on FL-DCs and GM-DCs was determined. Both cytokine production and maturation were assessed. FL-DCs treated with combinations of Poly I:C plus R848, or CpG ODN plus LPS appeared to produce more IL-10 than IL-12p70, while, similar amounts of IL-10 and IL-12p70 were released when FL-DCs were treated with Poly I:C plus LPS (Fig. 4.4B). In contrast, when FL-DCs were stimulated with a combination of CpG ODN and Poly I:C, more IL-12p70 than IL-10 was secreted (Fig. 4.4B). The amounts of IL-12p70 secreted by FL-DCs and GM-DCs activated with combined CpG ODN and Poly I:C were significantly augmented compared with treatment with CpG ODN or Poly I:C alone (Fig. 4.4C and 4.4D).

Based on the effect of CpG ODN and Poly I:C on IL-12p70 secretion by FL-DCs and GM-DCs, the influence of this treatment on the expression of maturation markers was investigated. As shown below, CD86, CD40 and MHC class II molecule expression by FL-DCs and GM-DCs was not affected by the combination CpG ODN and Poly I:C in comparison with treatments with CpG ODN or Poly I:C alone (Fig. 4.5A and 4.5B).

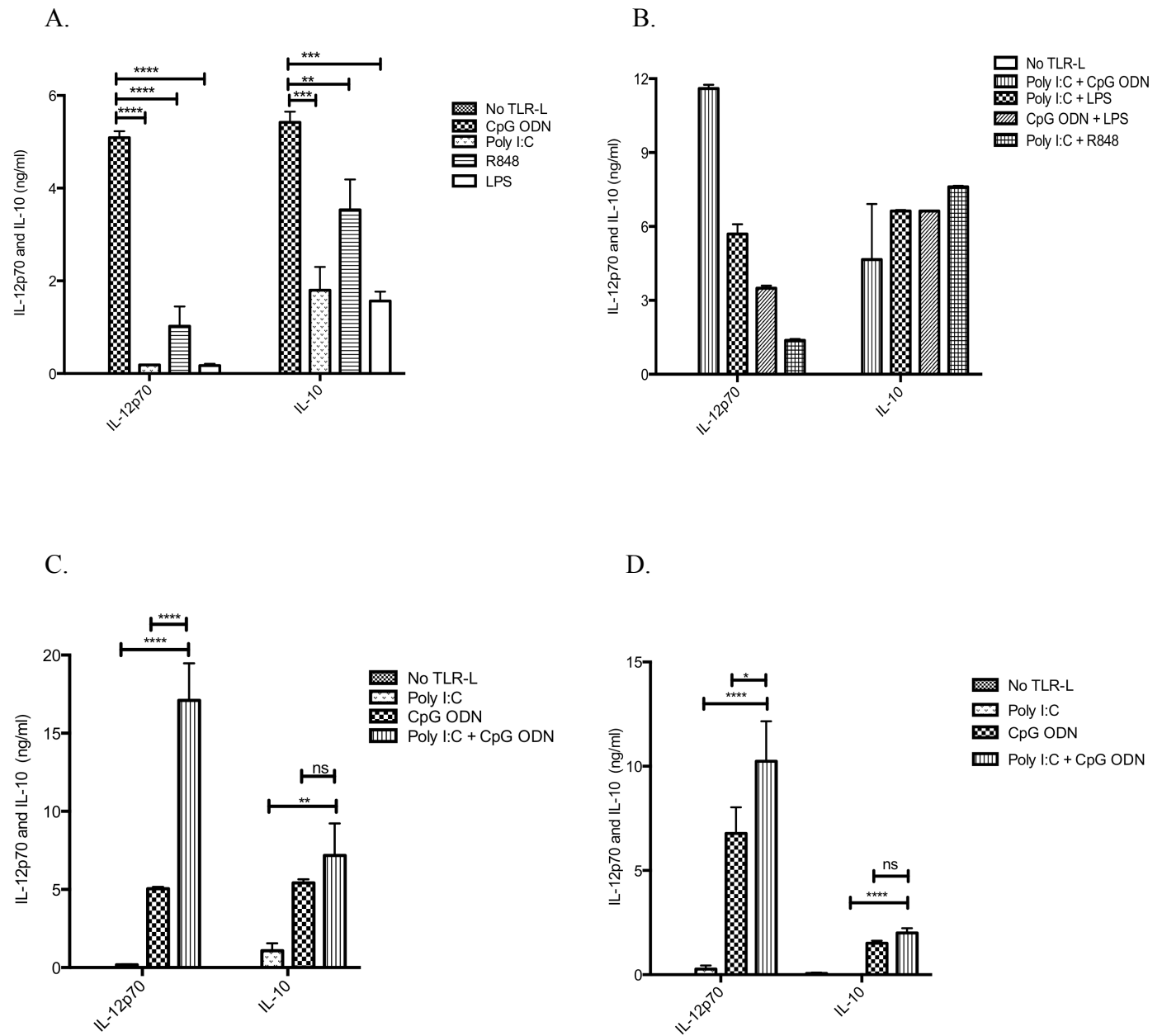


Figure 4.4

Figure 4.4. Stimulation with TLR-L(s) induces cytokine secretion by FL-DCs and GM-DCs. Day 8 (A, B and C) FL-DCs and (D) GM-DCs were collected, washed and cultured in the presence or absence of the indicated single TLR-L or combinations of TLR-Ls for 20 h. Supernatants were collected and the amounts of IL-12p70 and IL-10 secreted were determined by cytokine ELISA. Results represent mean with SEM of duplicates of (A) two and (C and D) three independent experiments (**** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, ns (not significant) $P > 0.05$).

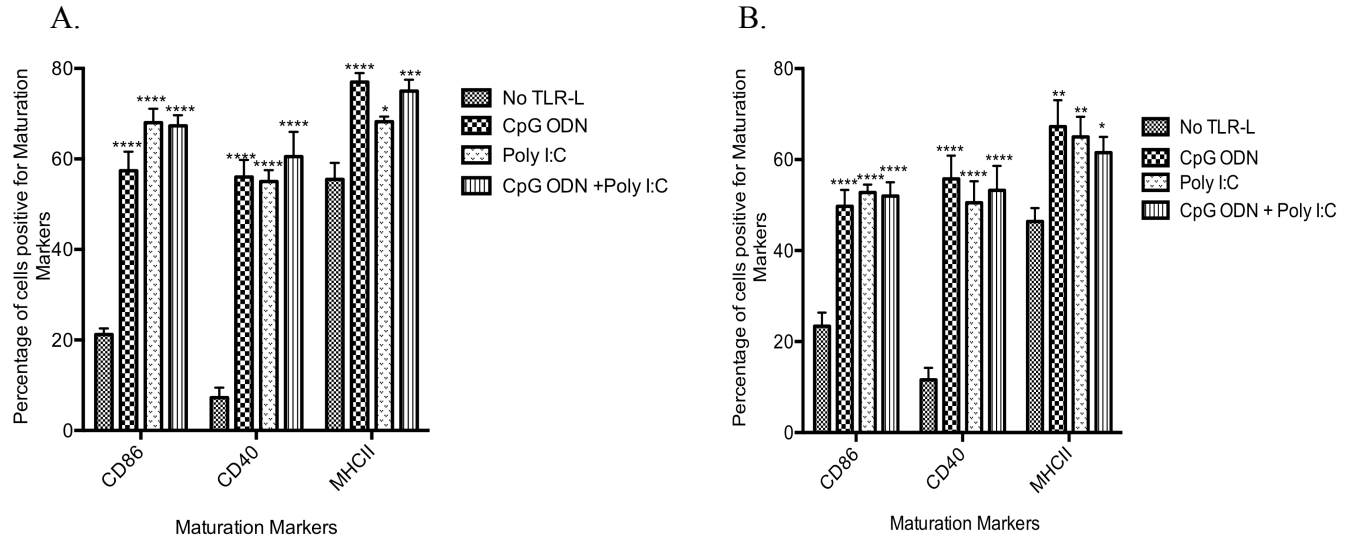


Figure 4.5. Combination of CpG ODN and Poly I:C does not affect the maturation phenotype of FL-DCs or GM-DCs. (A) FL-DCs and (B) GM-DCs collected on day 8 were left untreated or treated with CpG ODN, Poly I:C alone or a combination of CpG ODN and Poly I:C for 20 h. Cells were stained for maturation markers and the percentages of cells positive for these surface proteins were determined by flow cytometry. Mean with SEM from three independent experiments are shown. **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$: significance of difference between DCs treated with Poly I:C, CpG ODN or CpG ODN plus Poly I:C and DCs not treated with TLR-L(s). There was no significant difference between DCs treated with Poly I:C or CpG ODN and DCs treated with CpG ODN plus Poly I:C.

4.2.2 Allostimulatory capacity of FL-DCs

Mature DCs are capable of activating and inducing the proliferation of naïve T cells following antigen presentation. To assess the functional characteristics of matured FL-DCs, their immunostimulatory capacity was tested. TLR ligation on FL-DCs increased the level of expansion of allogeneic T cells when compared to the untreated FL-DCs and those cultured with autologous T cells (Fig 4.6A and 4.6B). Thus, TLR-activated FL-DCs had enhanced allostimulatory characteristics.

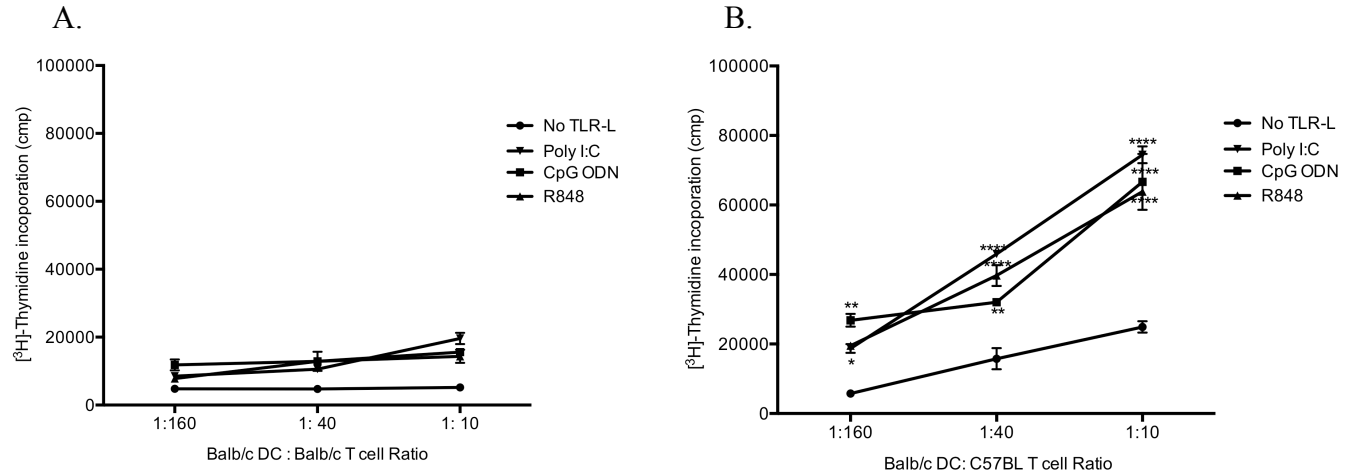


Figure 4.6. TLR ligand treated FL-DCs induce allogeneic T cell proliferation. Day 8 FL-DCs from Balb/c mice were left untreated or treated with CpG ODN (3 μ g/ml), R848 (500 ng/ml) or Poly I:C (25 μ g/ml) for 20 h. TLR-L-treated FL-DCs and untreated FL-DCs were cocultured with T cells obtained from splenocytes of (A) Balb/c or (B) C57BL mice for 3 days and pulsed with 3 H thymidine for 18 h. 3 H thymidine uptake by T cells in triplicate wells was then assessed by a scintillation counter. Mean with SEM of data is shown. ****P<0.0001, ***P<0.001, **P<0.01, *P<0.05: significance of difference between FL-DCs treated with Poly I:C, CpG ODN or R848 and FL-DCs not treated with TLR-L.

4.3 *Ex vivo* antigen loading of FL-DCs and GM-DCs

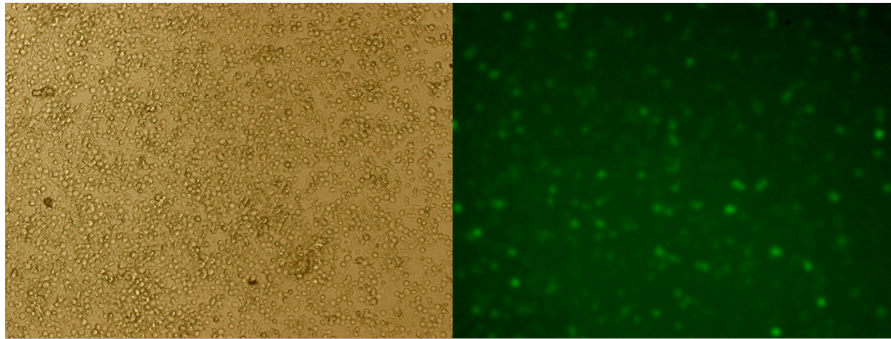
In DC-based vaccination, the number of DCs carrying the target antigen is crucial to the immune responses that would be elicited. Different non-viral methods of delivering mRNA to the FL-DCs to determine which one would lead to optimal transfection efficiency were examined. Transfection of FL-DCs with GFP mRNA by electroporation parameters E1 and E2 resulted in about 36% and 33% GFP positive cells, respectively. Depending on the electroporation parameters, greater than 80% of the cells were viable with E1 and E2 parameters when stained with trypan blue. In contrast, FL-DCs did not express GFP and only 50% cells were healthy after electroporation with E3 parameters. Also, transfection of FL-DCs using different Nucleofector programs yielded 12-18% GFP positive cells, and only about 50% of the cell population was still viable following nucleofection (Table 4.3.1). Based on the results obtained from this experiment, electroporation (E2) was used as a means of loading mRNA into FL-DCs in my subsequent work (Fig 4.7A). In contrast, transfection of GM-DCs with GFP mRNA by nucleofection resulted in 65% GFP positive cells with about 100% viability (Fig. 4.7B and 4.7C).

Table 4.1. Transfection efficiency and viability of FL-DCs 24 h post transfection with GFP mRNA

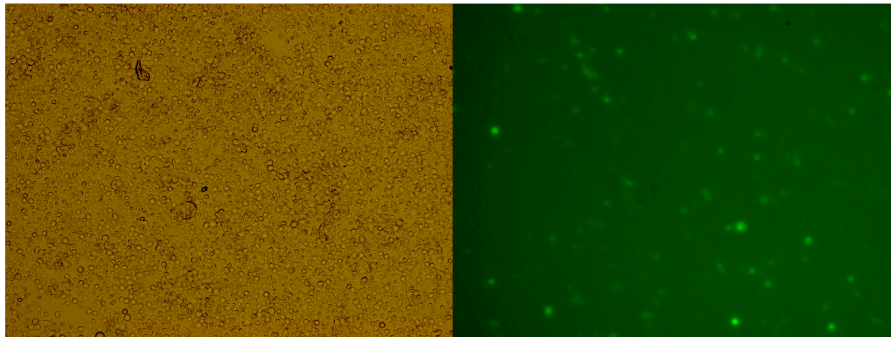
Transfection Method	Transfection Variables	Transfection Efficiency	Cell Viability 24 h post-transfection
Electroporation	300 V, 150 μ F, 8 msec (E1)	36%	80%
	350 V, 100 μ F 5 msec (E2)	33%	100%
	300 V, 300 μ F, 5 msec (E3)	No evidence of Transfection	50%
Nucleofection	K2, U2, X1, Y1	12-18%	50-57%

A.

Electroporation (300 V, 150 μ F, 8 msec) – 4×10^6 cells + 30 μ g eGFP mRNA



Nucleofector program Y1 - 4×10^6 cells + 30 μ g eGFP mRNA



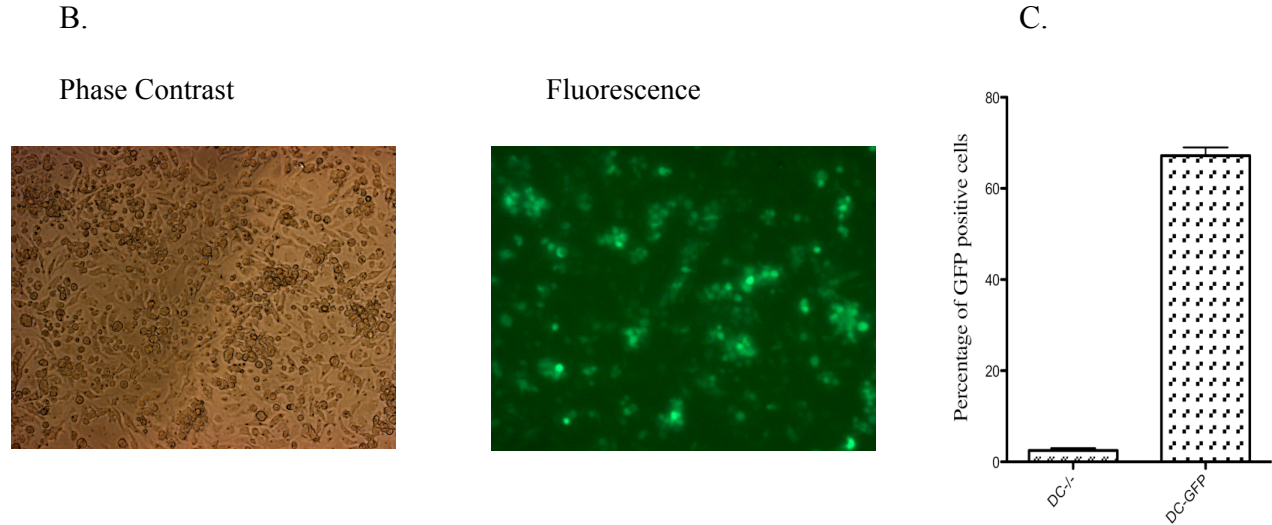


Figure 4.7. FL-DCs and GM-DCs loaded with GFP mRNA. FL-DCs or GM-DCs harvested on day 8 were electroporated or nucleofected with 30 μ g of GFP mRNA using the indicated electroporation parameters and nucleofector programs. Expression of GFP by (A) FL-DCs and (B) GM-DCs was observed under the fluorescent microscope. (C) Flow cytometry of GFP positive GM-DCs and the mean with SEM from two independent experiments is shown.

4.4 Functional characteristics of FL-DCs bearing HCV NS3/4A or NS5A used for vaccination.

To ensure that HCV mRNA transfected FL-DCs were potentially immunogenic, particularly those that were activated with CpG ODN, their functional properties were tested. FL-DCs electroporated with HCV NS3/NS4A or NS5A mRNA and treated with CpG ODN elevated the expression of CD86 and MHCII molecules compared to their untreated counterparts (Fig. 4.8A). FL-DCs transfected with HCV NS3/4A mRNA or left untransfected and treated with CpG ODN, stimulated proliferation of allogeneic T cells, compared to the unstimulated cells (Fig. 4.8B). Treatment of HCV antigen-bearing FL-DCs with CpG ODN induced the production of more IL-12p70 than IL-10 (Fig. 4.8C and 4.8D) unlike CpG ODN-activated FL-DCs that released equal amounts of IL-12p70 and IL-10 in the absence of exogenous antigen (Fig. 4.4A and 4.4B). In addition, transfection of FL-DCs with HCV NS3/NS4A or NS5A mRNA appeared to lead to IL-10 secretion in the absence of CpG ODN activation compared with untransfected FL-DCs (Fig 4.4B). I also demonstrated that FL-DCs transfected with NS5A mRNA expressed the HCV NS5A antigen by immunostaining (Fig. 4.9A and 4.9B)

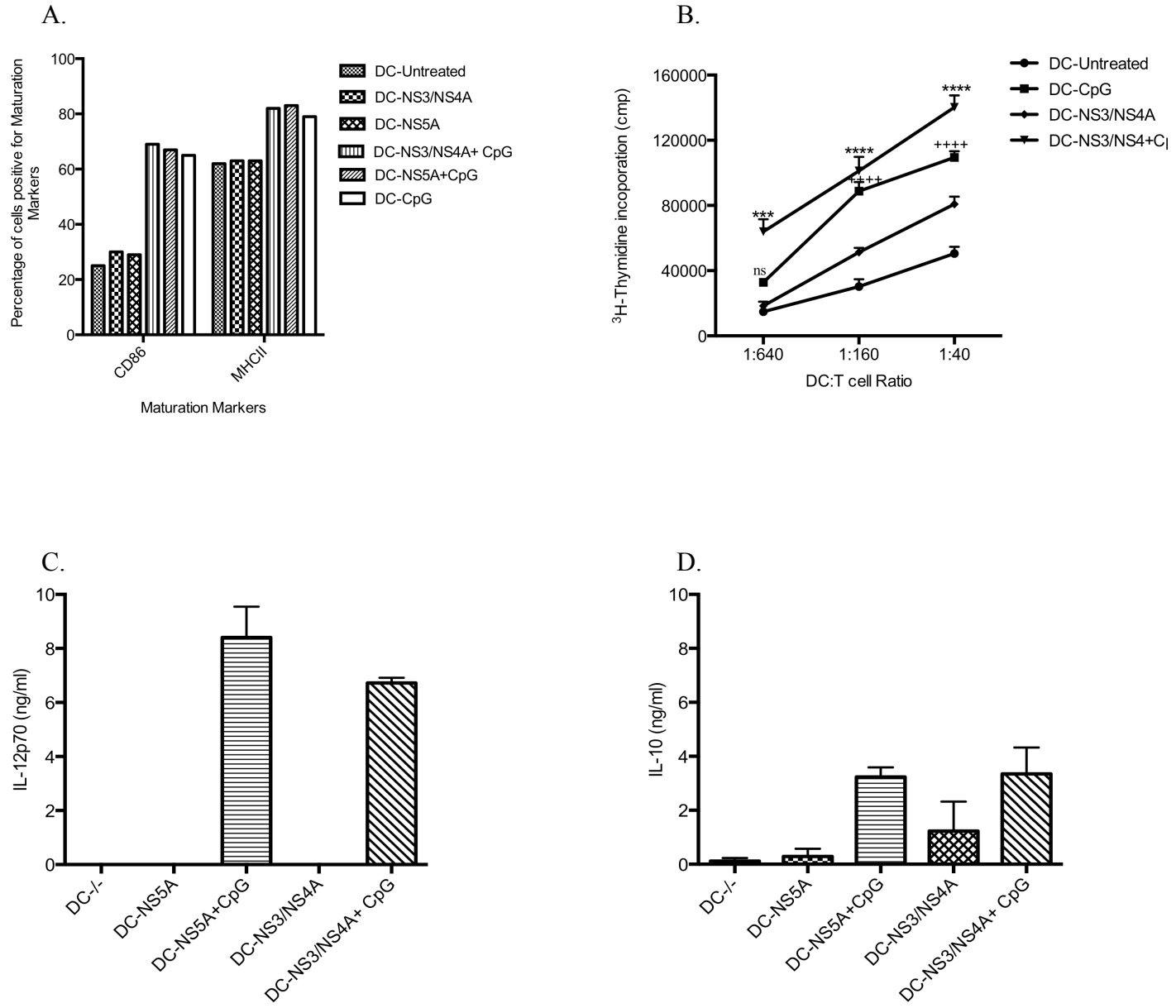
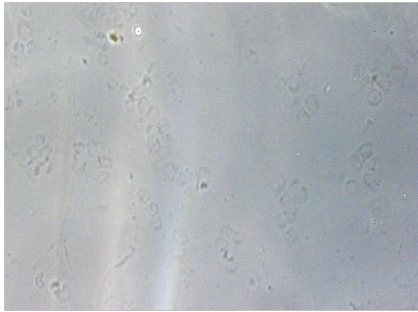


Figure 4.8

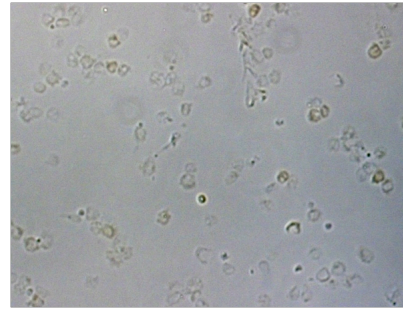
Figure 4.8. CpG ODN-activated FL-DCs *ex vivo* prior to adoptive transfer. Day 8 FL-DCs (DC-/-) were electroporated with HCV NS3/NS4A (DC-NS3/NS4A) or NS5A (DC-NS5A) mRNA, left untreated and/or treated with CpG ODN (3 µg/ml) for 20 h. (A) Cell surface proteins were stained and the expression of the indicated maturation markers was assessed by flow cytometry. (B) CpG ODN-treated or untreated FL-DCs were cocultured with T cells obtained from splenocytes of C57BL mice for 5 days and pulsed with ³H thymidine during the last 18 h. ³H thymidine uptake by T cells was then assessed by a scintillation counter. Mean with SEM from triplicates of one representative experiment of two is shown. ****P<0.0001, ***P<0.001: significant difference between FL-DCs bearing HCV NS3/4A left untreated and those treated with CpG ODN; ++++P<0.0001, ns P>0.05: significance of difference between untransfected FL-DCs left untreated and those treated with CpG ODN (C and D) Supernatants from the indicated FL-DC cultures were collected and used for cytokine ELISA. Mean with SEM from duplicates of one of two independent experiments is shown.

A.



DC-/-

B.



DC-HCV NS5A

Figure 4.9. Expression of HCV NS5A by FL-DCs. FL-DCs collected on day 8 were (A) left untransfected or (B) electroporated with 30 μ g NS5A mRNA. Expression of the HCV NS5A protein was determined by immunohistochemistry.

4.5 Adaptive immune responses and protection following immunization with FL-DCs transfected with HCV NS3/4A or NS5A mRNA.

HCV-specific T cell responses were determined by enumeration of IFN- γ and IL-5 ELISPOTS. Based on the results of this trial, there was no indication of HCV-specific cellular immune responses following stimulation of splenocytes obtained from HCV antigen-bearing FL-DC-vaccinated mice with recombinant NS3 or NS5A protein (data not shown). Accordingly, there was no significant alteration in the viral load in mice that received FL-DCs bearing HCV antigens as similar amounts of virus were recovered from the ovaries of the various treatment groups (Fig 4.10).

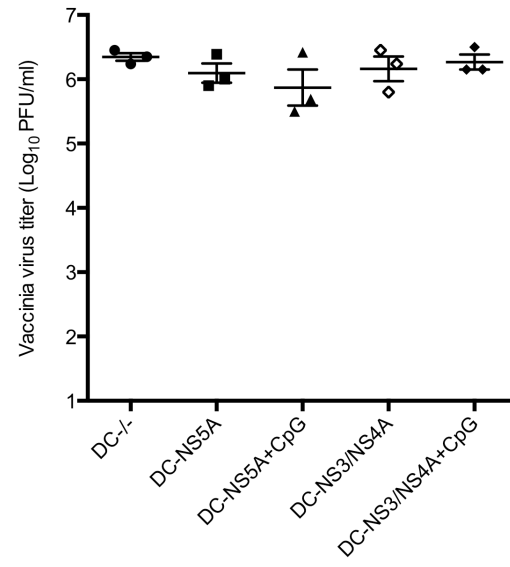


Figure 4.10. Recombinant vaccinia virus titer in challenged mice after immunization with FL-DCs bearing HCV antigens. Mice were challenged i.p with 5×10^6 pfu of rVV expressing HCV NS3/NS4/NS5 ten days after the last immunization. Five days after the virus challenge, the mice were euthanized to isolate ovaries, and rVV titers were determined on BSC-1 cells. Each data point represents an individual animal, and the mean with SEM indicated is indicated by horizontal and error bars.

4.6 Effect of route of administration and combination of CpG ODN and Poly I:C on immune responses after vaccination with FL-DCs transfected with HCV mRNAs.

To overcome the lack of protective immunity obtained in the previous trial, I set out to increase the numbers of mRNA-loaded FL-DCs used for adoptive transfer. In addition, various inoculation routes were examined as this has been reported to influence the immune responses after vaccination [246]. FL-DCs carrying HCV NS3/4A and NS5A antigens and treated with CpG ODN and Poly I:C were injected subcutaneously (s.c), intradermally (i.d), intravenously (i.v) or i.p into mice. The *ex vivo* stimulated mRNA-loaded DCs produced substantial amounts of IL-12p70 and were able to activate allogeneic T cell proliferation (Fig. 4.11A and 4.11B). Further, some of the mice vaccinated subcutaneously or intradermally elicited HCV-specific Th1-type responses based on the presence of IFN- γ -secreting cells with very few or no IL-5 secreting cells (Fig. 4.12A and 4.12B). However, there was no protection from rVV expressing HCV NS3/NS4/NS5 in any of the groups that received the DCs transfected with the HCV NS3/4A and NS5A mRNA (Fig 4.12C).

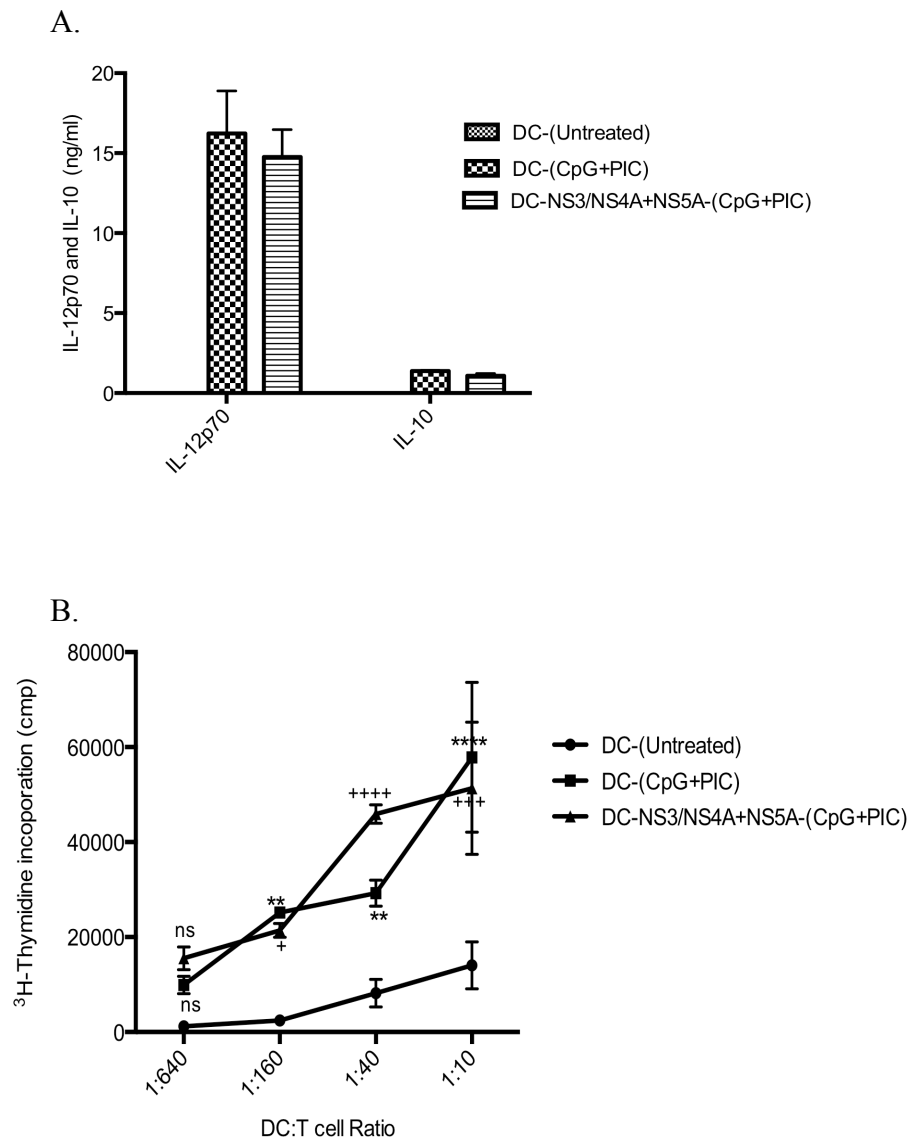


Figure 4.11

Figure 4.11. Cytokine secretion and allostimulatory capacity of CpG-ODN and Poly I:C treated FL-DCs carrying HCV antigens. FL-DCs were loaded with HCV NS3/NS4 and NS5A mRNA by electroporation on day 8. (A) Cells were incubated for 6 h to allow expression of the indicated HCV antigens and then treated with CpG ODN (3 µg/ml) and Poly I:C (PIC) (25 µg/ml) for 18 h. Supernatants from CpG ODN- and Poly I:C-treated DC-NS3/4A+NS5A cultures were collected for cytokine ELISA. Data represent mean with SEM of duplicates of 2 independent experiments. (B) Antigen-loaded FL-DCs were cocultured with T cells obtained from splenocytes of C57BL mice for 3 days and pulsed with ³H thymidine for 18 h. ³H thymidine uptake by T cells in triplicate wells was then assessed by a scintillation counter. ++++P<0.0001, +++P<0.001, +P<0.05, ns P>0.05: significance of difference between FL-DCs bearing HCV NS3/4A/NS5A treated with CpG ODN plus Poly I:C and untreated DCs; ****P<0.0001, **P<0.01 ns P>0.05: significance of difference between untransfected FL-DCs left untreated and those treated with CpG plus Poly I:C ODN.

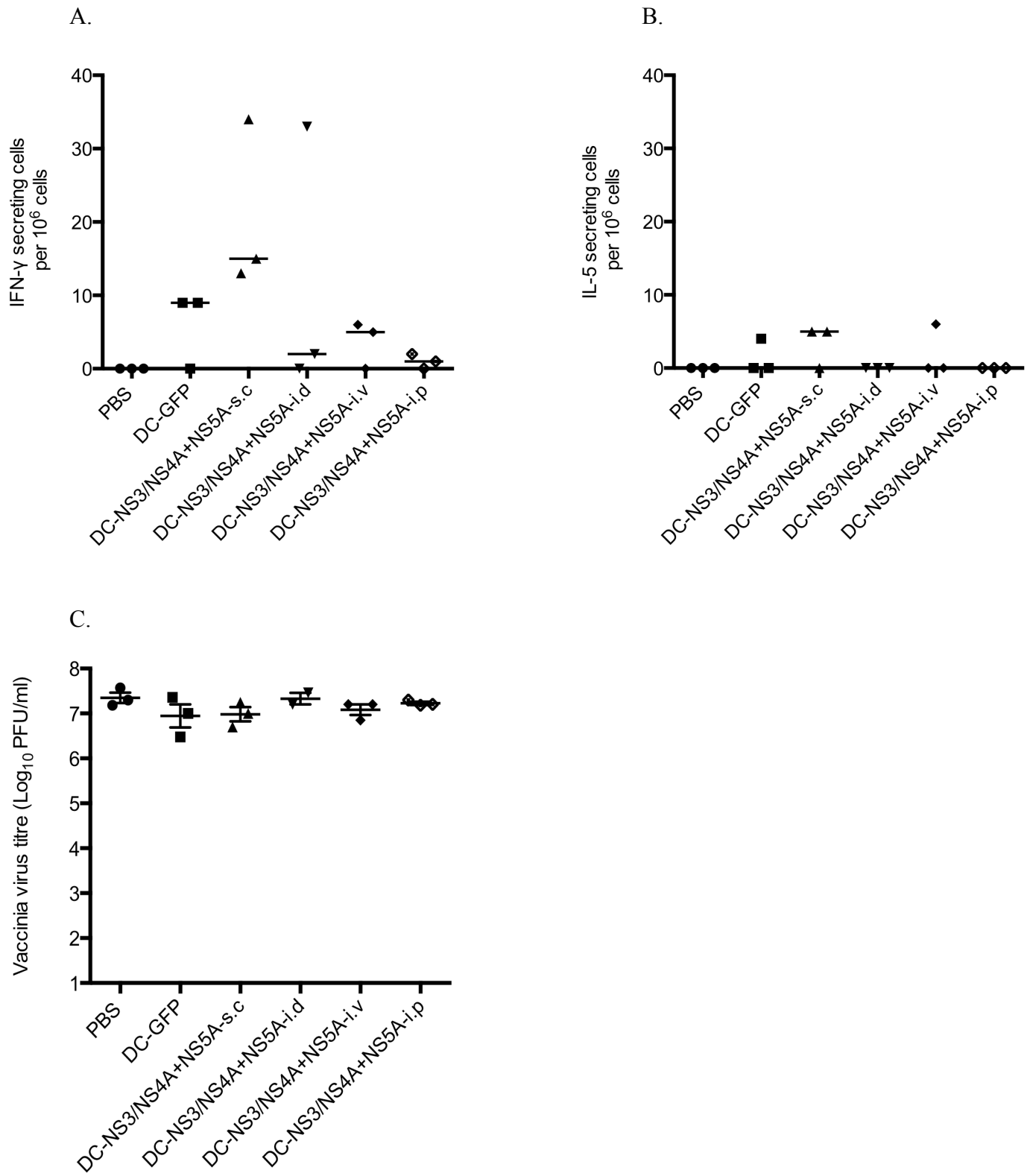


Figure 4.12. HCV-specific cell-mediated immune response and viral load following vaccination with HCV antigen-bearing FL-DCs. Balb/c mice were vaccinated with 1×10^6 CpG ODN- and Poly I:C-treated FL-DCs loaded with NS3/NS4A+NS5A mRNAs s.c, i.d, i.v or i.p. PBS and DC-GFP mice groups were injected s.c. Splenocytes were left untreated or stimulated with a combination of 1 μ g/ml of HCV rNS3 and rNS5A protein for the ELISPOT assay. The numbers of (A) IFN- γ and (B) IL-5 secreting cells were determined based on the difference in the number of spots between the antigen-pulsed and the mock-treated cells. Median values in (A and B) are shown by horizontal bars. (C) Ten days post-immunization, mice were challenged i.p with rVV encoding HCV antigens. Homogenates of ovaries were used to determine rVV titers in BSC-1 cells. Each data point represents a single animal. Mean with SEM of values in (C) are indicated by horizontal and error bars. There was no significant difference between the treatment groups.

4.7 Adoptive transfer of GM-DCs loaded with single or combined HCV mRNA(s) and activated with TLR-L(s)

Since weak or no HCV-specific responses were induced in the vaccine trial reported in 4.6, I examined whether a different type of DCs (GM-DCs) might improve the magnitude of the antigen-specific responses elicited after vaccination. Based on functional phenotypes, GM-DCs expressing HCV NS3/4A and/or NS5A and further stimulated with CpG ODN or a combination of CpG ODN and Poly I:C upregulated the expression of costimulatory proteins and MHC class II molecules (Fig 4.13A). The activated DCs stimulated the proliferation of heterologous T cells (Fig. 4.13B), and also released higher amounts of IL-12p70 and IL-10 (Fig 4.13C and 4.13D) when compared to the untreated cells *ex vivo*. Similar to what was shown earlier, CpG ODN and Poly I:C enhanced IL-12p70 secretion by GM-DCs bearing HCV antigen(s) compared to activation with CpG ODN alone. GM-DCs transfected with HCV mRNA(s) expressed the HCV antigen(s), as shown for NS3 (Fig. 4.14A and 4.14B). HCV-specific IFN- γ and IL-5 were induced in some of the mice that were administered CpG ODN-activated GM-DCs bearing HCV NS3/4A or NS5A. Nonetheless, there was no significant difference in the number of IFN- γ and IL-5 spots (Fig. 4.15A and 4.15B) and the viral burden (Fig. 4.15C) between the groups that received the DCs transfected with the HCV mRNA(s) and the GM-DC-GFP or PBS groups.

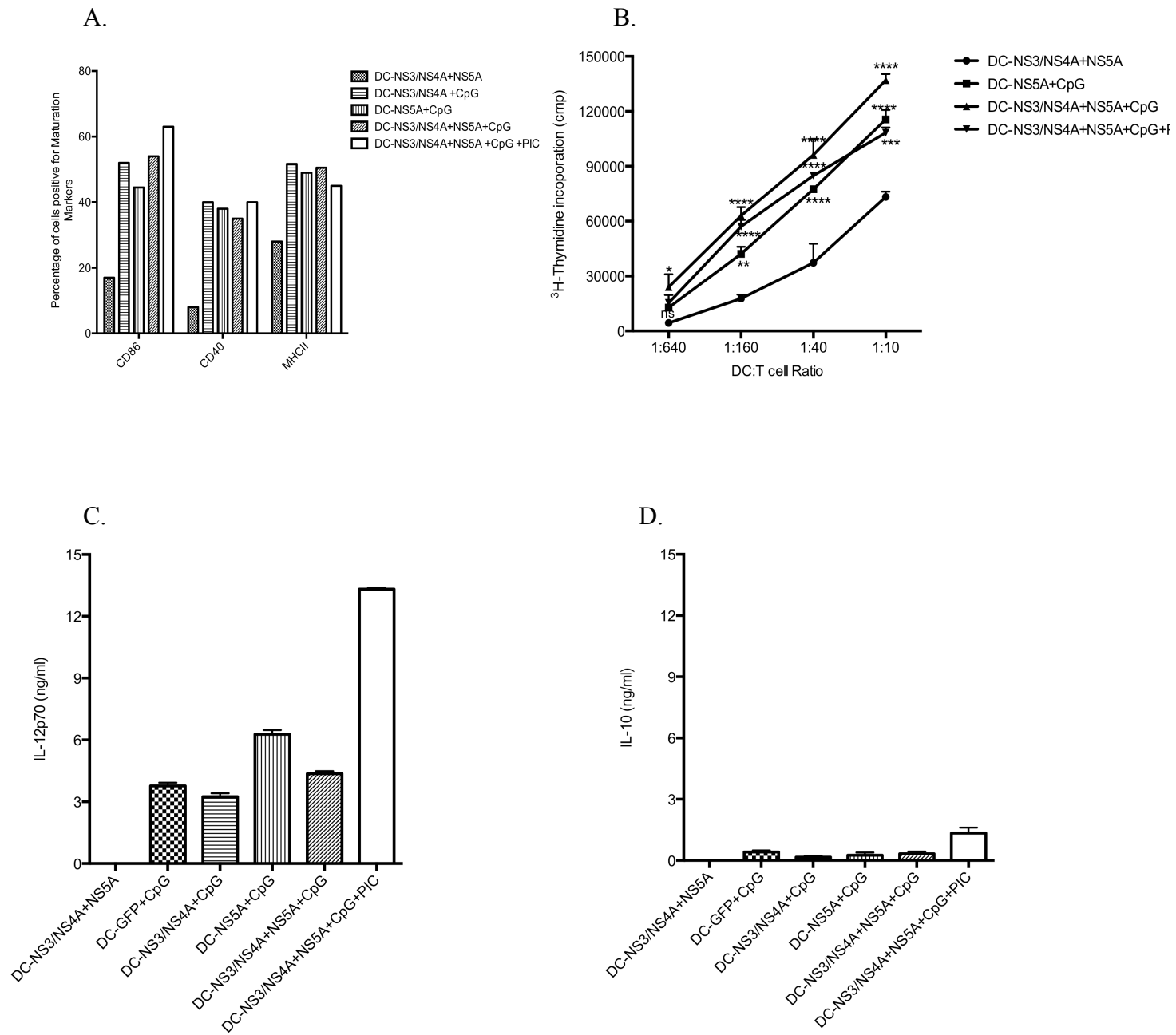
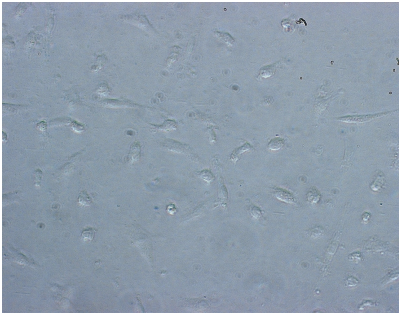


Figure 4.13

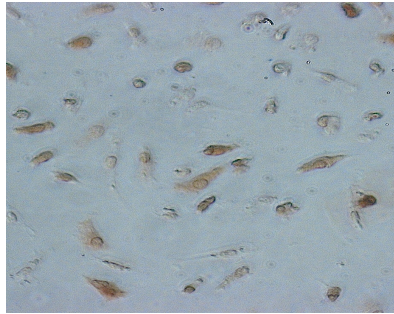
Figure 4.13. Impact of TLR-L(s) activation on GM-DCs used for adoptive transfer. Day 8 GM-DCs were nucleofected with HCV NS5A (DC-NS5A) or NS3/NS4A (DC-NS3/NS4A) or a combination of NS5A and NS3/NS4A (DC-NS3/NS4A+NS5A) mRNA. Cells were left untreated or treated with CpG ODN- or CpG ODN (3 µg/ml) plus Poly I:C (PIC) (25 µg/ml) for 20 h. (A) Expression of the indicated maturation markers was assessed by flow cytometry. (B) TLR-L(s)-treated or untreated GM-DCs carrying HCV antigen(s) were cocultured with T cells obtained from splenocytes of C57BL mice for 5 days and pulsed with ³H thymidine during the last 18 h. ³H thymidine uptake by T cells was then assessed by a scintillation counter. Mean with SEM from triplicates of one representative experiment of two are shown. ****P<0.0001, ***P<0.001, **P<0.01, *P<0.05, ns P>0.05: significant difference between FL-DCs bearing HCV antigens treated with CpG ODN or CpG ODN plus Poly I:C and those not stimulated with TLR-L(s). (C and D) Supernatants from the indicated GM-DC cultures were collected for cytokine ELISA. Results are shown as mean with SEM from duplicates of one representative of four separate experiments.

A.



DC-/-

B.



DC-NS3

Figure 4.14. Immunocytochemistry of GM-DCs expressing HCV antigen. GM-DCs collected on day 8 were (A) left untransfected or (B) nucleofected with 30 μ g of HCV NS3/4 mRNA. Expression of NS3/4A was identified with mAb specific for NS3.

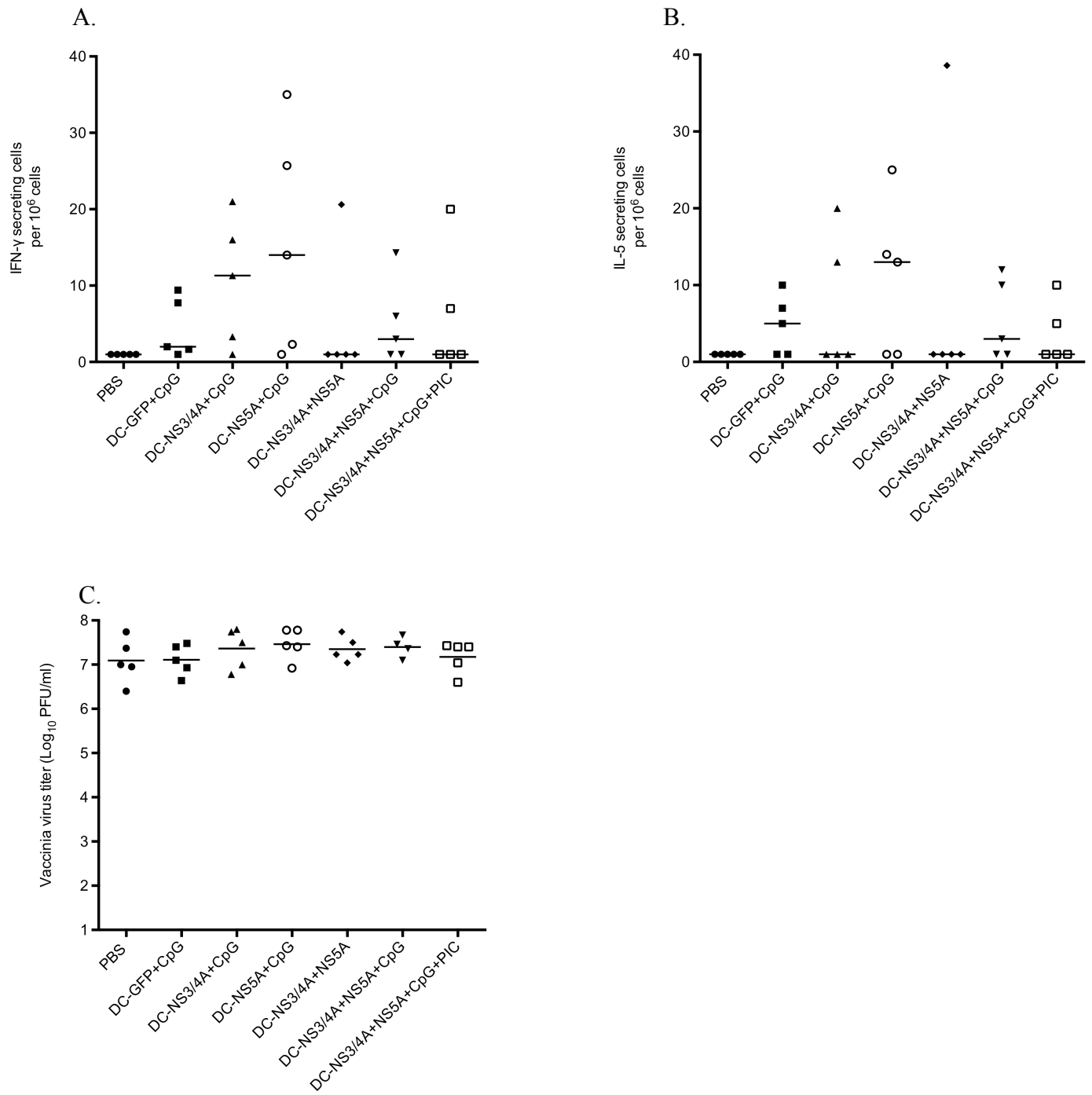


Figure 4.15

Figure 4.15. Effect of TLR-L(s) and HCV antigen(s) on adaptive immune responses and protection by GM-DC-based vaccination. The number of splenocytes secreting HCV-specific (A) IFN- γ and (B) IL-5 in mice given the different DC-vaccines, were determined by the difference in the number of spots between the antigen-pulsed and the mock-treated cells. Median of data are indicated by horizontal bars. (C) Recombinant vaccinia virus titers in the ovaries of vaccinated mice 5 days post challenge were determined on BSC-1 cells. Each data point represents a single mouse and the mean values are indicated by the horizontal bars. Similar results were obtained in another experiment.

4.8 CpG ODN-activated GM-DCs carrying HCV antigen secrete IL-5

IL-5 ELISPOT results from GM-DC vaccine studies revealed an abundance of non-HCV-specific IL-5 in groups that received GM-DCs bearing GFP or HCV antigen(s). I sought to investigate the source of this innate IL-5 as this might influence the immune responses induced. CpG ODN-stimulated GM-DCs expressing GFP or HCV NS5A, or left untransfected, produced increased IL-5, and not IFN- γ , when compared with untreated GM-DCs (Fig. 4.16A). Co-culturing these activated DCs with T cells, on the contrary, induced large amounts of IFN- γ rather than IL-5 in a MLR assay (Fig. 4.16B). Furthermore, after vaccination with the DCs tested above, no innate IL-5 spots were detected from a CD3-negative cell population (data not shown), while splenocytes containing CD3-positive T cells non-specifically secreted IL-5 and IFN- γ .

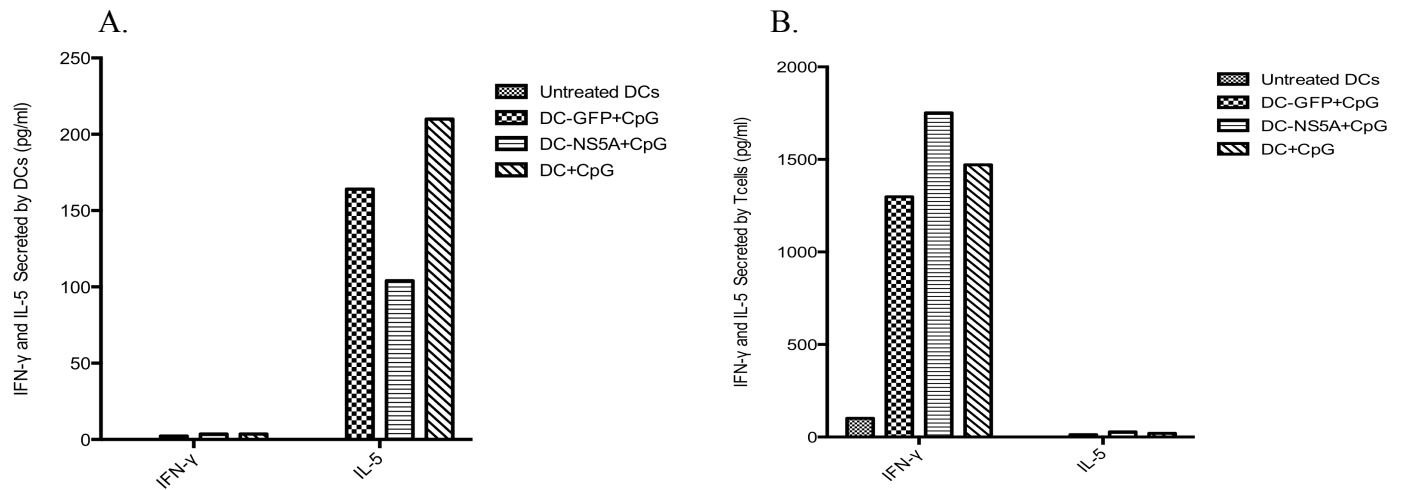


Figure 4.16. IFN- γ and IL-5 production in DC cultures and DC-T cell cocultures. GM-DCs loaded with GFP, NS5A mRNA and untransfected GM-DCs were stimulated with CpG ODN or left untreated. (A) The levels of IFN- γ and IL-5 released in the indicated DC cultures or (B) the DCs cocultured with allogeneic T cells were determined by cytokine ELISA. One of two independent results is shown.

4.9 HCV-specific immune responses induced by HCV recombinant NS3 or NS5A protein formulated with triple adjuvant.

Finally, I adapted a vaccination approach currently being used in my lab to see if I could induce robust HCV-specific responses with this strategy. Immunizing mice with HCV rNS3 or rNS5A formulated with CpG ODN, host defense peptide and PCEP produced significantly increased titers of antigen-specific antibodies in the serum with a near balance in IgG1 and IgG2a responses (Fig. 4.17A). Significant HCV-specific IFN- γ spots were observed from mice vaccinated with rNS5A compared to those administered rNS3 (Fig. 4.17B). However, no IL-5 and CD8⁺ IFN- γ ⁺ T cells were induced (data not shown) and no significant change in virus titers was observed in these vaccine groups compared to the control animals (Fig. 4.17C).

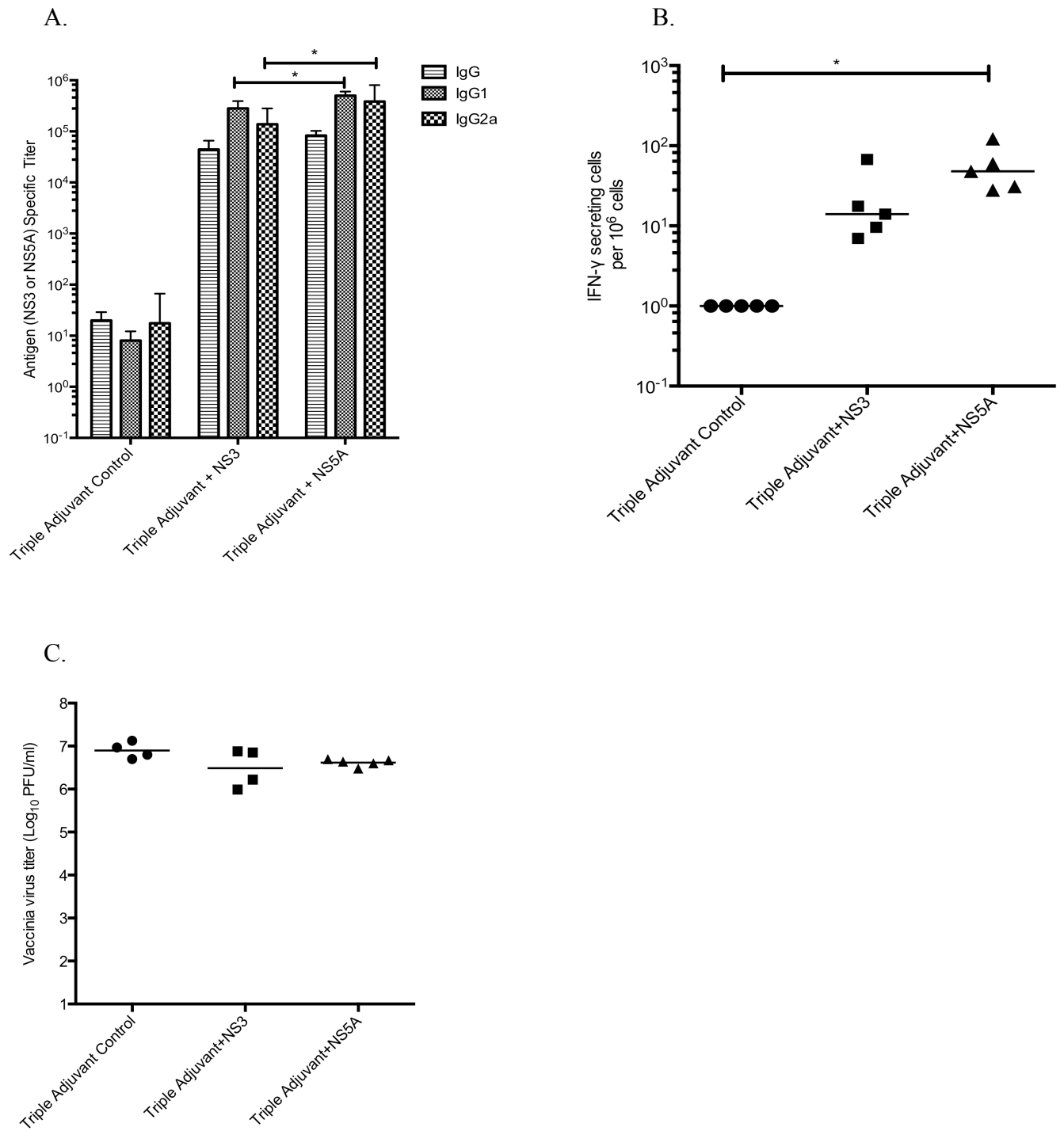


Figure 4.17

Figure 4.17. HCV-specific immune responses and viral load in mice vaccinated with rNS3 or rNS5A. Mice were immunized with PBS or HCV rNS3 or rNS5A formulated with triple adjuvant. (A) Serum IgG, IgG1 and IgG2a and (B) the numbers of antigen-specific IFN- γ producing splenocytes were assessed ten days after the second immunization. (C) Amount of rVV expressing HCV antigens present in the ovaries of vaccinated mice infected with the virus. Each data point represents a single mouse and the (B) median and (C) mean values are indicated by the horizontal bars. *P<0.05

5.0 DISCUSSION AND CONCLUSIONS

5.1 Impact of TLR agonist(s) treatment on the phenotypes of FL-DCs and GM-DCs

At present, GM-CSF and Flt3L are the major cytokines used to differentiate DCs from their precursors, for many species. Differential responses induced by FL-DCs and GM-DCs have been well documented. These responses are possibly correlated to specific features of these DCs. I demonstrated that FL-DCs derived from bone marrow cultures consisted of two subsets that resembled steady state lymphoid tissue-resident cDCs and pDCs [220]. GM-CSF signaling via STAT5 possibly inhibited pDC commitment and/or terminal maturation and thereby distinctively promoted almost an entirely cDC phenotype [245].

The goal of DC vaccination is to initiate potent protective T cell responses. The two key characteristics required for this to occur are the maturation and activation of DCs. Previously, the DCs generated by Yu *et al.* [226] and used in a DC-based vaccine against HCV probably were not fully matured which may have impacted the downstream partial protective immunity. Thus, this work was concentrated on priming the DCs to promote Th1 effector cell differentiation *in vivo*. The results from this study showed that, in spite of their DC subtype composition, the FL-DCs and GM-DCs were equally activated in terms of their morphology and the surface expression of CD86, 40 and MHC class II proteins, when exposed to synthetic TLR agonists

In addition, it was demonstrated that these DCs were capable of inducing T cell expansion by testing their allostimulatory capacity. However, by definition of cytokines released by FL-DCs and GM-DCs following treatment with CpG ODN, these might possibly lead to the induction of a balanced Th1/Th2 and polarized Th1 response,

respectively, subsequent to T cell differentiation. Furthermore, the cytokine profile of TLR3, TLR4 or TLR7-activated FL-DCs may facilitate Th2 development or the expansion of T regulatory cells [247]. While CpG ODN-activated FL-DCs have been reported to secrete more Th1 promoting cytokines, R848 induced more IL-10 than IL-12p70 similar to what was observed previously [248]. Increased production of IL-10 when compared to IL-12p70 by TLR-L-activated DCs has been attributed to signaling through p38 mitogen-activated protein kinase (MAPK) [249], and the time point fresh DCs obtained from cultures meet a maturation stimulus [250]. The former effect was reversed with p38 MAPK inhibitors [249]. However, the major players responsible for the differences in the cytokine profile between my results and the reported data, are not clear due to the variability in the generation of the FL-DCs, type of TLR-L and the duration of treatment [248, 250].

Strikingly, it was noted that when FL-DCs loaded with HCV mRNA were treated with CpG ODN, this favored the release of the Th1-promoting cytokine IL-12p70, in contrast to the results obtained in the absence of an exogenous antigen. The molecular mechanism for this is not known. However, ds secondary structures of mRNA delivered by lipofection have been reported to cause maturation and type 1 IFN production by monocyte-derived DCs. With this knowledge, it is possible that ds structures of the HCV mRNA may be activating TLR3 or RIG-1 [251] of FL-DCs, which are sensors of these kinds of molecular patterns. This perhaps indicates that the Th1-polarized *ex vivo* response exerted by CpG ODN-stimulated FL-DC loaded with HCV mRNA may be due to a synergistic response from TLR9 signaling by CpG ODN with either TLR3 or RIG-1 effects [252]. While the HCV mRNAs are single stranded and might mimic a TLR7

ligand, TLR9 does not seem to synergize with TLR7 [238], which thus is not likely the reason for driving CpG ODN-activated FL-DC transfected with HCV mRNA towards a Th1 promoting phenotype.

My results further showed significant cooperation between TLR9 and TLR3 signaling via combined CpG ODN and Poly I:C activation. This synergistic effect augmented IL-12p70 secretion by FL-DCs and GM-DCs, but did not impact the maturation status of the DCs. Again, this confirms the outcome of a similar study performed with GM-DCs [238]. Moreover, the data suggest that DC stimulation by the combination of these TLR-Ls may be advantageous when compared to the use of a single TLR-L due to their involvement in the activation of superior CTL responses and DC survival as reported in previous studies [236, 239]. DCs can also be activated with other inflammatory mediators, but these DCs are only able to support T cell proliferation and not their differentiation into Th1 or Th2 cells [253]. Essentially, the use of TLR ligands for DC activation used in these studies was appropriate.

5.2 HCV-specific immune responses induced by DC-based vaccination

Hepatitis C virus infections mostly lead to persistence of the virus and may progress into liver cancer. The ability of the virus to survive in its host affects the functions of very important immune cells including DCs. Since vigorous Th1 and CTL responses are needed to clear HCV, targeting DCs in therapeutic HCV vaccine development is paramount considering their multifunctional role in immunity.

Besides the activated state of DCs, one other critical parameter considered in vaccination strategies targeting DCs is the number of antigen-bearing DCs. Thus, to

induce strong HCV-specific T cell responses in this study, I had to be able to efficiently load the DCs with HCV mRNA. In a receptor-mediated endocytosis study it was revealed that GM-DCs were more efficient in capturing antigen in comparison with FL-DCs [189]. In agreement, I showed that GM-DCs excelled in capturing mRNA when compared to transfection of FL-DCs. This suggests that fewer numbers of antigen-bearing GM-DCs may be required for priming T cells in vaccination compared to FL-DCs.

In this study, the first vaccine trial with FL-DCs loaded with HCV NS3/4A or NS5A mRNA, left untreated or treated with CpG ODN, no HCV-specific Th1 responses or protective immunity was induced. However, when the numbers of HCV mRNA-loaded FL-DCs were doubled in the second trial, depending on the route vaccination, antigen-specific Th1 responses were induced in some of the animals. This further confirms the need for increased numbers of mRNA-loaded DCs when FL-DCs are used as vaccine vectors as they are not very proficient at capturing antigen. It was surprising that despite doubling of the numbers of FL-DCs carrying antigens, either no or weak adaptive HCV-specific immune responses were elicited. In addition, the latter supports the lack of protection, as robust cell-mediated immunity is required to clear the virus infection. Moreover, the route of vaccine inoculation of the *ex vivo* Th1-polarized FL-DCs did not significantly influence the immune responses contrary to what was anticipated or has been proposed [246]. In contrast, DCs such as pDCs and CD8 α^+ cDCs, which can be generated with Flt3L, have been implicated in poor antigen presentation [254], T cell stimulation [255] and T cell tolerance [201]. Thus, although I did not extensively study the subtypes of FL-DCs, it is still possible that the composition of the

FL-DCs may be involved in my inability to induce strong Th1 HCV-specific protective immunity *in vivo*.

There is evidence that GM-DCs are superior in activating anti-tumor immunity when compared to FL-DCs [256]. This, together with my results from the FL-DC-based vaccinations, prompted me to use GM-DCs as an alternative vehicle. The functional features of *ex vivo* GM-DCs transfected with HCV NS3/4A and/or NS5A mRNA, and treated with CpG ODN or Poly I:C or a combination of the two TLR agonists, demonstrated that these cells were Th1-polarized and capable of priming naïve T cells. Thus, the GM-DCs were activated optimally to induce potent antigen-specific response and possibly a stronger response *in vivo* following vaccination with HCV antigen-bearing GM-DCs stimulated with double TLR-L. However, no or weak HCV-specific Th1 responses induced with GM-DCs carrying HCV antigens were elicited similar to what was observed with FL-DCs. These responses were unable to alter the viral burden upon infection. Hence, activating protective immunity by combining HCV mRNAs and TLR-Ls was not realized. In the absence of TLR-L treatment, GM-DCs loaded with HCV NS3/4A or NS5A mRNA, were described previously to be potent in generating HCV protective immunity [175, 226]. In the latter studies, induction of an average of about 170 IFN- γ -secreting cells per million splenocytes corresponded to significant reduction of viral titers or protection. This indicates that the T cell responses elicited in my study were weak based on the mean number of antigen-specific IFN- γ secreting cells induced. Although the GM-DCs used in the previous studies were relatively mature compared to those in this study, it not clear why the DCs that received maturation stimuli were not capable of inducing similar immunity. In addition, vaccination with DCs transduced with

adenovirus (Ad) encoding HCV NS3 did not only induced Th1 responses, but also protected mice from tumor cells expressing HCV NS3 and reduced the expression of HCV RNA in the liver [179]. Furthermore, DCs transduced with Ad expressing TLR5 ligand and SOCS1 siRNA, pulsed with HCV E2 antigen were superior in inducing both antigen-specific cellular and humoral immune responses, when compared to DCs transduced with Ad expressing TLR-5 agonist or SOCS1 siRNA alone [257].

It has been shown that DCs can get “exhausted” in their ability to produce cytokines later after stimulation and have the capacity to activate naïve T cells to nonpolarized “central memory” T cells which lack immediate effector function, instead of effector Th1 or Th2 cells [258]. The latter could be a possibility in the T cell responses induced in my vaccine trials. However, the *ex vivo*-primed DCs used for adoptive transfer appeared to not be exhausted after acquiring immunostimulatory features when they were activated with TLR-L(s), because they activated T cells in the 5-day MLR assay and produced Th1-polarizing cytokine. In addition, when HCV antigen-bearing DCs were left untreated with TLR-L, no protective immunity was induced.

Furthermore, I suspected that innate IL-5 might be altering HCV-specific immune responses generated. I showed that activating the GM-DC cultures with CpG ODN also induced IL-5 besides IL-12p70 and IL-10. The former is probably an activity of granulocytes [259, 260] as GM-CSF also promotes differentiation of granulocytes that can respond to CpG ODN [261, 262]. Little or no IL-5 was, however, produced when the GM-DCs were co-cultured with allogeneic T cells. Also, CD3-negative T cells were not responsible for secreting what appeared to be innate IL-5, and thus granulocytes may not be entirely responsible for the innate response induced. Moreover, non-specific IL-5 and

IFN- γ were induced in almost equal amounts in my subsequent vaccination study. This could be a vaccination-associated effect and may have nothing to do with the weak HCV-specific responses produced. Thus, it remains to be resolved why I was unable to generate robust adaptive immunity with the DC-vaccination strategy.

5.3 Cellular and humoral responses to HCV recombinant protein vaccine

There is an urgent need for a HCV vaccine. Current results from my preclinical studies with DC-based vaccines against HCV infection did not show great promise, thus the need to test other vaccine strategies. Alternative vaccines in development against HCV include recombinant protein vaccines. This vaccine approach serves well as both prophylactic and therapeutic vaccine as compared to DC-based vaccines, which are preferably being developed as immunotherapies. So far, the outcomes of HCV recombinant protein vaccines tested in clinical trials have been quite promising. While these vaccines induce both HCV-specific humoral and cellular immunity, in one of the first studies, in the face of chronic HCV infection, the responses elicited did not change the progression of liver disease in patients [182].

Most subunit or recombinant protein-based vaccines including those tested with HCV antigens fail to produce strong antigen-specific responses when the antigens are used alone [263, 264]. Therefore, the need to co-formulate the antigens with some modifiers to enhance immune responses induced is necessary. The components of the triple adjuvant used in this work were carefully selected to target a number of immune factors to induce an optimal response. Synthetic CpG ODNs as shown by the Th1-associated effects on DCs are known to help skew immune responses towards a Th1-

biased or a balanced response [265, 266]. Cationic host defense peptides on the other hand are small peptides with antimicrobial and immunomodulatory properties. Also, they induce secretion of chemokines that support the recruitment of monocytes, T cells, DCs, among others [267]. Finally, polyphosphazenes, poly [di(sodiumcarboxylatophenoxy)-polyphosphazene] (PCPP) and poly [di(sodium carboxylatoethylphenoxy)-polyphosphazene] (PCEP) belong to a class of synthetic, water-soluble macromolecules with a phosphorus-nitrogen backbone [268]. Polyphosphazenes can be used in a microparticle formulation [259] and also have immunostimulatory properties and prolong antigen retention capabilities [268, 269].

In this vaccine trial, it was demonstrated that formulating HCV rNS3 or rNS5A protein with triple adjuvant induced both HCV-specific antibody and Th1 cell responses. Between the two proteins tested, the HCV-specific IFN- γ response was found to be greater against rNS5A than to rNS3. The immune responses induced, however, did not lead to protection during surrogate infection with rVV expressing HCV NS3/NS4/NS5.

Considering the heterogeneous nature of HCV, the virus proteins that are relatively conserved will be necessary in formulating a HCV recombinant protein vaccine as this allows the generation of immune responses against a wide range of viral strains in infected persons. Unlike the hypervariable envelope proteins, the HCV core and nonstructural proteins have more conserved regions [182]. In addition, in chronic HCV infection, HCV core, NS3, NS4B and NS5A were found to be the major immunogenic antigens among the HCV proteins [270], thus, supporting the suitability of the choice of HCV NS3 and NS5A as antigens.

Several preclinical studies with HCV rNS3 and rNS5A proteins have been performed in the last few years. In contrast to my data, recently, it was reported that HCV rNS5A formulated with adjuvant induced a mixed Th1/Th2 response with a robust Th2 component due to low levels in IFN- γ secretion; however, a Th1 pathway was favored when a heterologous prime-boost (rNS5A-plasmid NS5A) method was used [271]. While I was unable to induce protection in the vaccinated mice after infection, possibly due to the absence of IFN- γ -producing CD8⁺ T cells, in an earlier study where mice were immunized with *Mycobacterium bovis* Calmette–Guerin bacillus (BCG) expressing a single CTL epitope of HCV NS5A, effector CD8⁺ T cells were induced. This led to significant viral reduction, following challenge with rVV encoding the HCV NS5A gene [176]. It is, however, noteworthy to mention that NS5A binds to MyD88, an adaptor of TLR 9, and prevents the recruitment of interleukin-1 receptor-associated kinase 1. Cytokine and chemokine release is consequently down-regulated upon activation with CpG ODN [272]. Therefore, it is possible that the immune response induced with NS5A and the triple adjuvant may have been a modulated response. Furthermore, when HCV polyprotein composed of core, NS3, NS4 and NS5 was used in another vaccination study, little or no IFN- γ ⁺-CD8⁺ T cells were generated. No protection assay was, however, carried out, so I am unable to compare the efficacy of their vaccine with my results [263]. HCV rNS3 has also been shown to generate Th2 or balanced Th1/Th2 responses, as well as strong long-lasting CTL and Th1 responses [264, 273, 274].

The major differences found between this study and those reported above are the amount of antigen (which ranged from 1.4 μ g to 25 μ g) and more especially the type of adjuvant used in the vaccine formulation. These may have accounted for some

similarities and differences between my test vaccines and others. For instance, 4 months after immunization with HCV rNS3 vaccine formulated with poly I:C, the percentages of CD4⁺ IFN- γ ⁺ and CD8⁺ IFN- γ ⁺ T cells were higher than when CpG ODN was used. However, similar T cell responses were obtained in the two groups 7 months after vaccination [274]. In addition, depending on the composition of adjuvants, the immune system is activated in several ways and this also alters the type of immune response induced [274]. On the contrary, when 1 μ g of respiratory syncytial virus F protein was co-formulated with the triple adjuvant both antigen-specific adaptive response and complete protective immunity were induced [275].

5.4 General conclusion and future directions

In summary, this study was carried out to improve upon the efficacy of protective immune responses to HCV using an existing DC-based vaccine strategy. I assessed immune responses and protection in mice immunized with *ex vivo* derived DCs loaded with HCV NS3/4A and/or NS5A mRNA and treated with TLR agonist(s). My data showed that both bone marrow-derived FL-DCs and GM-DCs activated with CpG ODN or CpG ODN plus Poly I:C and loaded with HCV mRNA(s) had a mature phenotype and were Th1-polarized. Stimulation with CpG ODN plus Poly I:C, however, resulted in preferential augmentation in IL-12p70 release by the DCs compared to CpG ODN or Poly I:C alone. Activated FL-DCs or GM-DCs carrying HCV antigen(s) elicited weak or no HCV-specific adaptive immune responses in mice, and therefore were unable to clear rVV expressing HCV NS3/NS4/NS5.

Furthermore, it was shown that HCV rNS3 or rNS5A formulated with triple adjuvant, CpG ODN, host defense peptide and PCEP, induced a balanced antigen-

specific Th1/Th2 immune response. HCV rNS5A induced a stronger T cell response than rNS3. Again, the HCV-specific responses did not reduce the viral load in infected mice.

In my hands, the use of HCV recombinant protein vaccine showed more promise than the DC-based vaccine approach. Thus, in the future, the components of the recombinant protein vaccine may have to be optimized to help induce a strong cytotoxic T cell response required in viral clearance. Also, further studies may be needed to determine the vaccine dose and how the immune response with multiple antigens compares to a single HCV antigen.

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